ABSTRACT OF THESIS

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Degree Doctor of Philosophy *Date* 25th. August, 1965.

Title of Thesis The Molecular Size and Shape of Some Starch-Type Polysaccharides.

Physical techniques have been applied to the study of the starch polysaccharides, amylose and amylopectin, and glycogen; these include ultracentrifugation, viscosity, light-scattering and nuclear magnetic resonance.

The behaviour of amylopectin solutions has been shown to be determined by two effects. The first, at low concentrations ($< 2 \times 10^{-3}$ g./ml.), is due to the phosphate groups present in amylopectin and confers typical polyelectrolyte behaviour on the amylopectin molecule. The second effect, which occurs at higher concentrations, is apparently the formation, by some of the amylopectin molecules, of colloidal aggregates. Amylopectin has been converted to its "free acid" form by ion-exchange resins.

The results of present studies on starch biosynthesis are inconsistent with the current biosynthetic theories. From a consideration of the literature and the present work a new theory has been proposed.

Studies on the extraction of glycogen from liver, by various methods, have shown that the broadest distribution of molecular sizes are extracted by a technique which uses a phenol/water system rather than the classical 30% sodium hydroxide solution, or some more modern methods. The effect of degrading agents upon glycogens of various sizes has been studied by the effect of these agents upon the sedimentation-coefficient distributions of differing glycogens. Molecular-weight distributions have been derived for three broad distribution glycogens and their β-amylolysis dextrins, and these indicate that all the molecular weight species of the glycogens are attacked to the same extent by the enzyme. A "sub-unit" hypothesis has been proposed for high molecular weight glycogen.

Studies /

Use other side if necessary.

Studies on the N.M.R. spectroscopy of glucose oligomers have shown that the conformation of the glucose monomer in a long chain polymer such as amylose is unaffected by alkaline conditions (0.15M - 4M) or by the addition of salt of the dilute alkali. The gross hydrodynamic changes which occur under these conditions are ascribed to greater freedom of rotation about the glycosidic α-1 4 bond. It is considered likely that the conformation adopted is C1. The same conclusions have been shown to apply for dimethylsulphoxide solutions of the oligomers.

Number-average molecular weights have been derived from light-scattering measurements on unfractionated amylose solutions.

The action pattern of three α-amylases has been compared by light-scattering measurements of their action on various substrates. The rates of enzyme attack on branched substrates relative to amylose have been predicted from a consideration of the number of sites on the polysaccharide molecules available for attack by the enzyme.

A THESIS

submitted for the degree of

DOCTOR OF PHILOSOPHY

by

ROBERT GEDDES, B.Sc.

UNIVERSITY OF EDINBURGH. 25th. August, 1965.

TO MY PARENTS

and

ESTELLE.

GENERAL INTRODUCTION.

Starch is the most abundant reserve carbohydrate in plant sources where it occurs in the form of discrete granules. It can be fractionated into two polymers of D-glucose - the linear amylose and the branched amylopectin.

In contrast to starch, glycogen, the reserve glucan of animals, is distributed throughout the protoplasm and is not morphologically differentiated into granules. It is, however, closely related structurally to the starch polysaccharides. Differences in the degree of branching of the three substances affects their physical properties greatly and by studying these properties some of the finer points relating to differences in structure can be elucidated.

Although much work has been carried out on both starch and glycogen, it is only comparatively recently that any clear understanding of their structure has been obtained. Many problems, as will be shown in Section I, remain unsolved and the work of the author has been devoted to an attempt to make progress in this direction.

OUTLINE OF THESIS

Section I outlines the nature of starch and glycogen and outlines recent advances in their chemistry, whilst the next Section contains details of the experimental techniques used by the author. The results obtained for the aqueous solution behaviour of amylopectin are discussed in Section III, and are followed in the next Section by a new theory of starch biosynthesis. This theory accounts for the experimental biosynthetic results obtained for amylopectin. Section V is devoted to studies on glycogen, its mode of isolation, and its subfractionation. Some hydrodynamic behaviour of amylose is discussed in Section VI and Section VII is concerned with the nuclear magnetic resonance of starch oligomers and their possible bearing on the conformation of the glucose units in the starch polymers. The rate of attack of the enzyme α-amylase on various substrates is discussed in the last Section.

ACKNOWLEDGEMENTS.

I wish to gratefully acknowledge the help and encouragement given to me by Dr. C.T. Greenwood. I wish also to thank Professor Sir Edmund Hirst and Professor T.L. Cottrell for the provision of laboratory facilities and the Department of Scientific and Industrial Research for the provision of a grant.

CONTENTS.

CONTENTS (Continued)

SECTION I

GENERAL INTRODUCTION.

SECTION I

A. STARCH.

The nature of the molecular architecture of the granule is closely related to the theories of starch biosynthesis and is therefore discussed in Section IV. Quantitative separation of the two polymers from their heterogeneous mixture in the granule was not achieved until 1942 (Meyer).

Most native starches have been found to contain ca. 20% amylose. This is an essentially linear polymer where the α-D-glucopyranose units are linked through the 1 and 4 positions. Molecular weights are generally of the order of 10^5 to 10^6. The major component of starch, amylopectin, has a linear 1→4 linked backbone like amylose but which is interspersed with 1→6 links (ca. one per 20-30 monomer units) conferring on it a branched structure. Its molecular weight is generally of the order of 10^7 to 10^8. Various theoretical structures have been proposed for amylopectin and the most conclusive evidence to date, based on the enzymic degradation studies of Larner et al (1952), suggest that the "ramified tree structure" (Meyer and Bernfeld (1940)) is the most likely.

Before fractionation into its two components, the potato starch used throughout this thesis was pretreated with liquid ammonia (Hodge et al (1948)). Other pretreatments can be used (See, e.g., Banks et al (1959)). The starch was then dispersed in boiling water /

water with vigorous stirring in a nitrogen atmosphere for one hour to form an approximately 0.5% solution. After cooling to 60°C, thymol was added. The amylose-thymol complex, formed after standing for 48 hours at room temperature, was removed by high-speed centrifugation. The product was then redispersed and recrystallised as the butan-1-ol complex. Amylopectin was obtained from the supernatant liquor by freeze-drying after removal of thymol by extraction with ether.

Since the chemistry of the two components has been reviewed relatively recently (Greenwood (1956), Whelan (1958)) reference will only be made to the more recent advances.

Amylose

Early chemical evidence for the linear nature of amylose was apparently confirmed by the finding that amylose was completely converted into maltose by the action of the enzyme, β-amylase. Peat et al (1949) later showed, however, that with completely pure enzyme only ca. 80% of the available amylose is converted into maltose, and only when an impurity, Z-enzyme, was also present, did the hydrolysis approach 100% conversion. These results indicated that amylose was not completely linear and later workers (Cowie and Greenwood (1957), Arbuckle and Greenwood (1958), Banks et al (1959)) have shown that there are two types of amylose molecules present in the starch granule:

(a) completely linear amylose

(b) amylose with some form of structural anomaly which prevents its complete degradation by β-amylase.

A/

A recent suggestion as to the nature of this anomaly was that it might be due to the modification of the C_6 hydroxyl of some of the glucose monomers to aldehydic or carboxylic groups. (Banks et al (1959)). It was further suggested that the modification may be introduced during fractionation. Banks and Greenwood (1961) consider it unlikely that the modification is due to ester-phosphate groups.

Kjølberg and Manners (1963) have presented enzymic information which confirms the suggestion of Greenwood (1960) that the anomaly might be due to a small amount of branching in some amylose molecules.

Amylose has a pronounced tendency to precipitate out of aqueous solutions (retrogradation). This is a complex phenomenon and is dependent upon a number of factors, including pH, concentration of the amylose solution, and the molecular size of the amylose. (Foster and Sterman (1956), Paschell and Foster (1952), Whistler and Johnson (1948), Loewus and Briggs (1957), Husemann et al (1963), Čeh and Vene (1959)). Retrogradation is apparently due to associative forces causing parallel aggregation of the long linear amylose chains until colloidal dimensions are exceeded and precipitation occurs (Meyer, (1942)).

A recent study (Rao and Foster (1963)) of the conformation of amylose in aqueous solutions by viscosity, optical rotation and light-scattering comes to the conclusion that in neutral solution amylose is a stiff coil with an essentially helical backbone. At high pH values (ca. 12) there is a partial breakdown of helical structure with a concomitant increase in backbone flexibility.

This /

This disagrees with Banks and Greenwood (1963) who found that amylose behaves in a random coil in 0.33M potassium chloride solutions in agreement with the results of Everett and Foster.

Amylopectin.

Although periodate oxidation has indicated that the great majority branch-linkages in amylopectin are of the 1⟶6 type, Hamilton and Smith (1956) have, by the isolation of glucose from periodate-oxidised starch residues, indicated that 1⟶2 or 1⟶3 links may be present. More recent work (Manners and Mercer (1963)) has failed to confirm these findings.

Roberts and Whelan (1960) have provided additional evidence for Meyer's "ramified tree" amylopectin structure by the isolation of an oligosaccharide, containing two branch points, from the products of α-amylase action on amylopectin.

Since the findings of Samec (1935) the appreciable (ca. 0.05%) phosphorus content of potato starch has been ascribed to the presence of ester-phosphate groups on the C_6 of the glucose monomer. Fukui (1958) has treated amylopectin and its β-limit dextrin with kidney phosphatase at pH9.2 in an attempt to remove the phosphate groups. It was found that, although the phosphatase hydrolysed completely the phosphorus groups present in glucose-6-phosphate only a few per cent of the phosphate groups present in amylopectin and its β-limit dextrin were hydrolysed under comparable conditions. It is concluded that the nature of the phosphate linkages in the polymers and the monomer are different (See also MacGregor (1964)).

The apparent disaggregation of amylopectin acetate as the concentration is lowered, which was shown by the osmotic pressure measurements /

measurements of Kerr et al (1951) have been confirmed by Stacy and Foster (1957). These authors have also measured the apparent sedimentation constant distribution for corn amylopectin. The distribution indicates the extreme molecular weight heterogeneity in amylopectin. This is also shown by the electrophoretic experiments of Lewis and Smith (1957).

Erlander and French (1958) have shown, from experiments on the rates of acid hydrolysis of corn amylopectin, that the large amylopectins apparently exist in aggregates of four smaller units. This must be chemical aggregation since they have also shown (Erlander and French (1957)) that physical aggregation in amylopectin cannot be destroyed by hydrogen bond-breaking solvents.

Statistical models for amylopectin and glycogen have also been proposed (Erlander and French (1956), Erlander and French (1958), Erlander (1959)).

B. GLYCOGEN.

Classically glycogen was always extracted from tissue by the method of Pflüger (1904) which involves digestion of the tissue with 20-60% aqueous alkali at 100°C for a number of hours. It is only comparatively recently that the extensive degradation of polysaccharide material by this method has been established (Stetten et al (1958), Stetten and Katzen (1961)). A different method, which employs ice-cold trichloroacetic acid (TCA) has been utilised during the last few decades (Sahyun and Alsberg (1930), Stetten et al (1956)). Extraction of glycogen has also been performed with hot water (Greenwood and Manners (1957)). The molecular weights (weight-average) of glycogens extracted by the above methods indicate that the hot water degrades to approximately the same extent as alkali, (molecular weight ca. 10^6) while TCA extracted glycogens are usually in the range $(50\text{-}100)\times10^6$. However it has also been pointed out (Orrell and Bueding (1958), Stetten and Stetten (1960)) that the TCA-extraction also results in marked degradation.

In an attempt to avoid degradation of "native" glycogen several milder extraction methods have been devised. They are:
extraction by (a) cold water (Bueding and Orrell (1961))
(b) dimethyl sulphoxide (Whistler and Bemiller (1962))
(c) phenol and water (Laslov and Margoliash (1963))
(d) glycine buffer (Bueding and Orrell (1964)).

The efficiency of these methods is discussed in Section V .

Molecular weight distributions have been evaluated by Bryce et al (1958)/

(1958) for glycogen and its β-amylolysis dextrin. Since the form of the distribution curve depends entirely on the relation between S, the sedimentation coefficient, and C, the concentration, and on the relation between S and the molecular weight, both of which are difficult to measure, great absolute accuracy of the molecular weight distributions has not been claimed. Indeed the S - C relation used by Bryce and his co-workers disagrees with that used by Larner et al (1956) and no direct comparison between the two distribution curves can be made.

Bueding and Orrell (1961, 1964) have avoided the absolute inaccuracy of this method by using sedimentation coefficient distributions and comparing their glycogen products relatively.

The nature of the alkaline degradation of polysaccharides has been extensively reviewed by Whistler and BeMiller (1958).

Glycogen has been fractionated by differential centrifugation (Holme et al (1958)), Jones (1959), Stetten et al (1958)) and also by precipitation with cold ethanol (Stetten et al (1958)). The former gives apparently better fractionation.

The molecular distribution of glycogen has also been shown to be a function of its metabolic environment (Stetten et al (1960), Bueding and Orrell (1961)).

SECTION II.

EXPERIMENTAL METHODS.

SECTION II.

EXPERIMENTAL METHODS.

INTRODUCTION - Polymer Molecular Weights.

Different molecular weights arise because of the different methods of molecular weight determinations which are applied to polydisperse systems. For example, since the osmotic pressure is proportional only to the number of units in solution irrespective of their weights, measurements by this method yield a number-average value. This is true of any colligative property. The light-scattering technique measures the weight-average molecular weight since the amount of light scattered is a function of the size of the molecule, and can be directly related to the molecular weight of the molecule.

Since all colligative properties are inversely proportional to the molecular weight, measurement of them directly is a hazardous procedure for the extremely high molecular weight substances such as the starch polymers and glycogen. It is much more satisfactory to obtain weight-average molecular weights from light-scattering measurements. Although viscosity measurements are relatively easily made, molecular weights on this basis only yield an ill-defined average, very dependent on the $[\eta]$-molecular weight relationship, and molecular weights derived from ultracentrifugal sedimentation and density measurements are also very ill-defined averages. However recent advances in techniques have enabled $\overline{M}_w$ and $\overline{M}_n$ to be evaluated, in appropriate circumstances (see, for example, Proctor (1965)).

II. a. LIGHT-SCATTERING.

The phenomena of the scattering of light is basically one of diffraction and since scattered light has, for the most part, the same wave-length as the incident light it can be easily distinguished from ordinary fluorescence. Useful information is obtained by measuring relative intensity, depolarisation, and the angular distribution of the scattered light, and analysing these quantities in terms of the electromagnetic theory of radiation and the kinetic theory of matter. The range of molecular sizes over which such calculations have been applied is tremendous. At the small end of the scale, Cabannes (1929) checked Avogadro's number with calculations based on the quantitative measurements of the intensity of light scattered by argon and Debye (1947) checked the molecular weight of sucrose in a similar manner. At the other end of the scale, the molecular weight and shape of particles as large as the wavelength of the light used have been examined.

The Rayleigh Concept.

Lord Rayleigh (1871) was the first to appreciate that light-scattering by particles small compared with the wavelength of the light was basically a diffraction phenomenon, and not caused by refraction, reflection, or fluorescence.

Upon encounter of an isotropic particle of sufficient optical density with a beam of light, the electrons of the particle, being charged units, are caused by the incident electromagnetic impulse to /

to vibrate in unison with it, an oscillating electric moment thus being induced in the particle. These oscillating electrons are the sources of the scattered light which, for the most part, has the same frequency as the exciting wave.

Scattered light is radiated from the particle at every angle and the incident beam is weakened in intensity accordingly. There will be variations in the intensity of the scattered light, depending on the angle at which it emanates from the particle, since the vectors of the induced electric moments are parallel to those in the exciting beam. As a result of this the light scattered at 90° to the incident beam will be plane-polarised regardless of whether the incident beam was polarised or not. Hence for unpolarised incident light, the vertically polarised component (with respect to the plane of observation) is scattered symmetrically, whereas the horizontally polarised component is scattered proportionally to $\cos^2\theta$ where θ is the angle of observation measured from the incident beam. This gives rise to the $(1 + \cos^2\theta)$-term in the Rayleigh scattering equation for the scattering of unpolarised incident light from a small isotropic sphere:

$$\frac{i_{\theta,r}}{I_o} = \frac{8\pi^4\alpha^2}{\lambda^4 r^2}(1 + \cos^2\theta) \qquad (1)$$

where $i_{\theta,r}$ is the scattered intensity at distance r from the scattering centre and at an angle θ to the incident beam.

I_o is the incident intensity

α is the polarisability

λ is the wavelength of the light.

For /

For a dilute solution of spherical particles, the scatter will be multiplied by the concentration and it can also be shown that

$$\alpha = \frac{M}{2 \Pi N} n_o \left(\frac{dn}{dc}\right)$$

where M is the molecular weight of the particles

N is Avogadro's Number

n_o is the refractive index of the solvent

and $\frac{dn}{dc}$ is the refractive index increment.

Since the concentration = number of particles per c.c.= $\frac{Nc}{M}$

where c = concentration in gm./c.c.

Then $\frac{i_{\theta,r}}{I_o} r^2 = \frac{2 \Pi^2 n_o^2 \left(\frac{dn}{dc}\right)^2}{N \lambda^4} . M.c \, (1 + \cos^2\theta)$ (2)

The ratio $\left\{\frac{i_{\theta,r}}{I_0} . r^2\right\}$ has become known as the Rayleigh ratio or the reduced intensity, and it is preferable to use this quantity, denoted by the symbol R_θ, since this is the quantity measured in most light-scattering methods (i.e. the excess scattering of solute over the solvent).

Extension of Large Molecules.

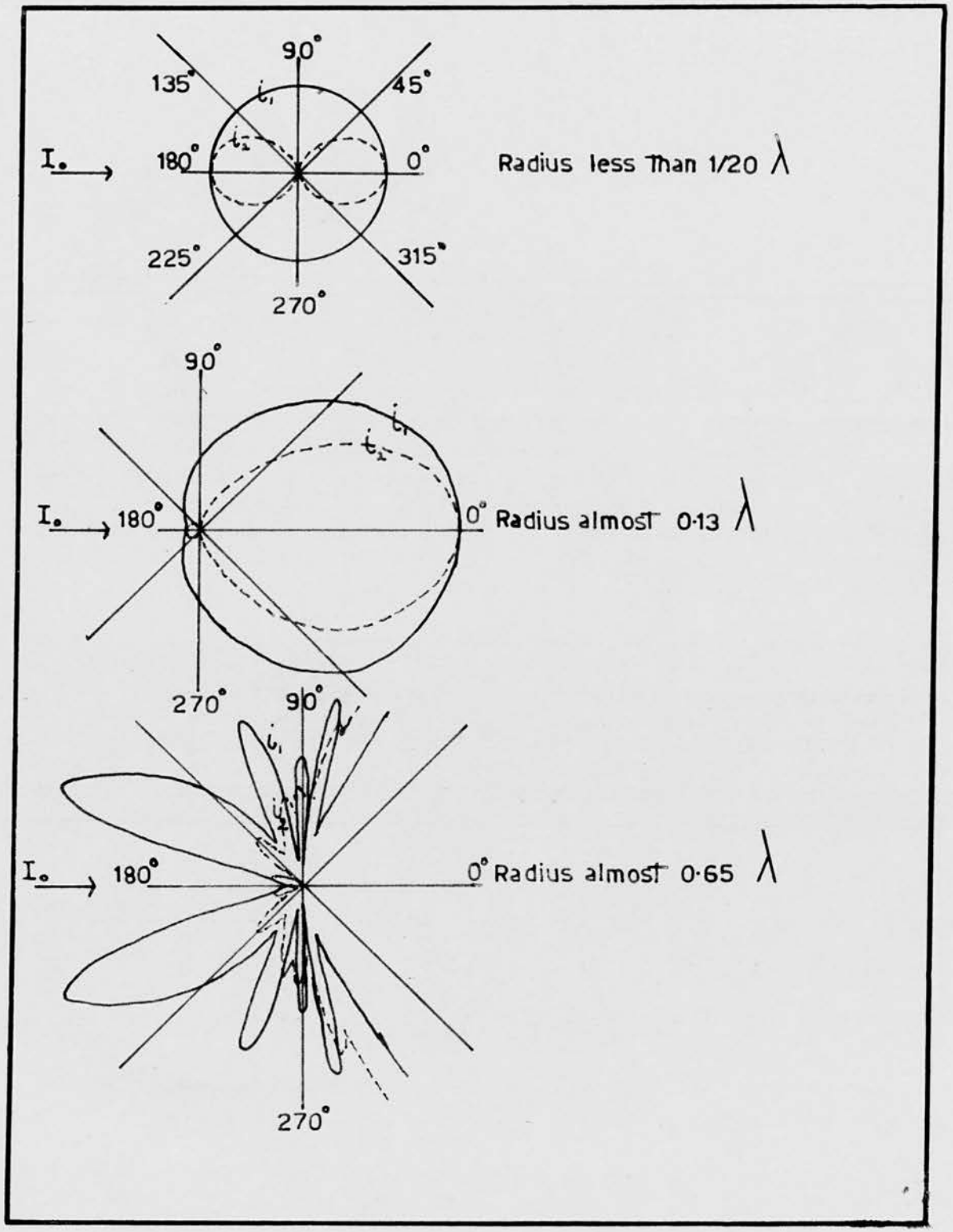
Debye (1947) extended the Rayleigh theory to dilute solutions of large molecules, by approaching the problem from a different viewpoint.

When light passes through a solution, it will lose intensity depending upon the degree of scattering. This can be expressed in a manner similar to the Lambert-Beer relation.

$$I = I_o e^{-\tau x}$$

where /

FIGURE 1



RELATIVE INTENSITIES of SCATTERING about ISOTROPIC SPHERES.
[After BLUMER (1925).]

where τ , the turbidity, can either be directly measured (spectrophotometer) or can be evaluated from the scattered light intensity, and X is the optical path length.

The need for calculating r, as in the Rayleigh equation, is eliminated.

In the ideal case, the turbidity is related to the Rayleigh Ratio at 90° by:

$$\tau = \frac{16\,\pi}{3} R_{90}$$

Hence

$$\tau = \frac{16}{3} KcM = Hcm \qquad (3)$$

where K and H are constants for the particular solution and wavelength and

$$K = \frac{2\,\pi^2}{N\lambda^4}\, n_o^2 \left(\frac{dn}{dc}\right)^2, \qquad H = \frac{16}{3} \cdot K$$

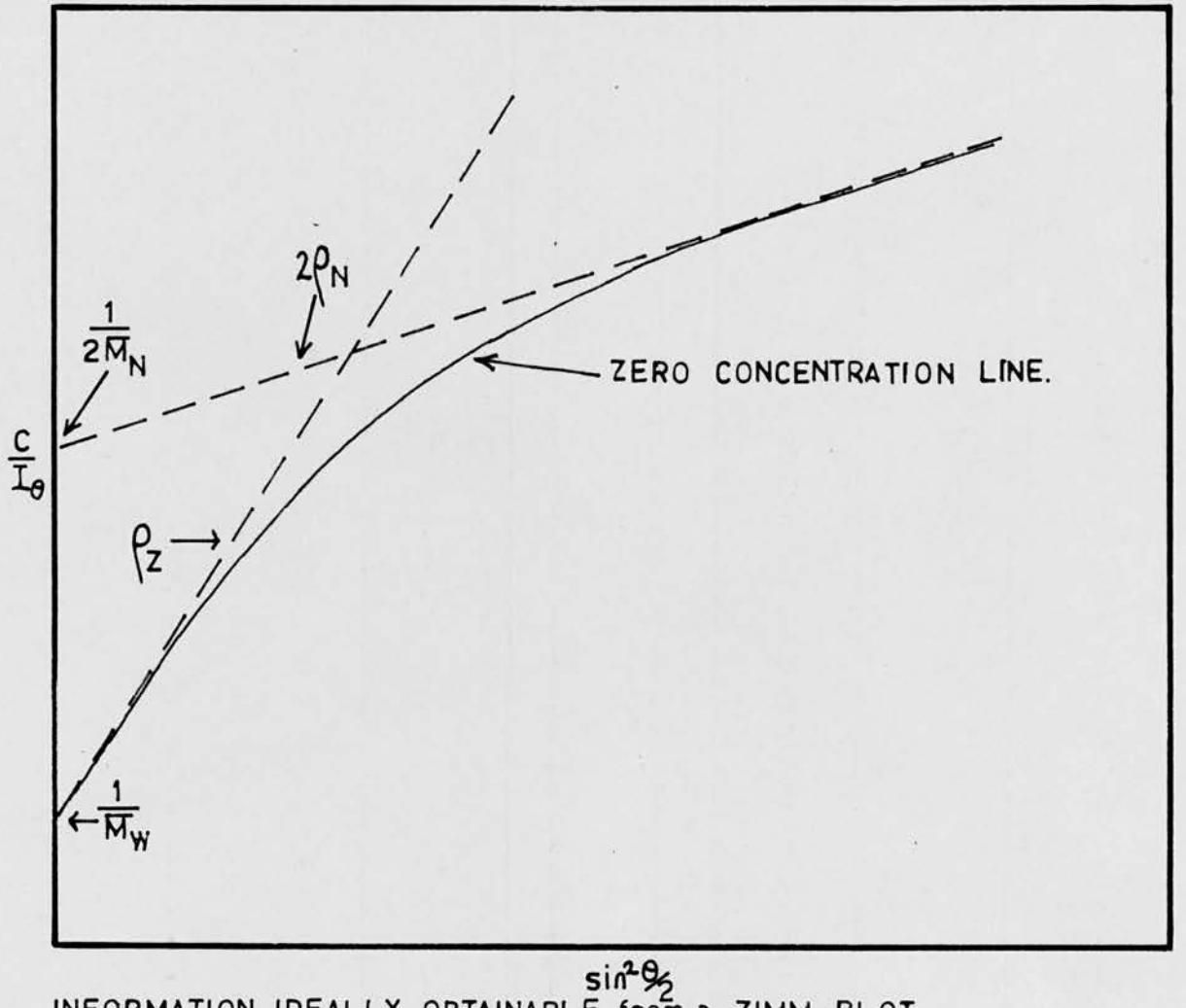
When particles are greater than $\frac{1}{20}\lambda$ in at least one dimension, the particles are large enough for the light scattered from one part of the molecule to interfere with that from another part and the scattering will no longer be symmetrical. This departure from symmetry will increase with particle size (see Figure 1) and is also found to be dependent on the actual shape of the particle. This effect has been calculated for several models e.g. the random coiling chain (Zimm (1958)), (Hermans(1958), Hyde et al (1958)).

The ratio of the observed intensity of a particular model to the intensity expected in the absence of interference is termed the Particle Scattering Factor, P_θ .

$$\text{i.e.} \quad P_\theta = \frac{(R_\theta) \text{ observed}}{(R_\theta) \text{ ideal}}$$

P /

FIGURE 2



INFORMATION IDEALLY OBTAINABLE from a ZIMM-PLOT.

P_θ equations for simple molecules are shown below

Spheres $$P_\theta = \left[(3/x^3) \cdot (\sin x - \cos x)\right]^2 \qquad (4a)$$

Coils $$P_\theta = (2/y^2) \cdot \left[\exp(-y) - (1-y)\right] \qquad (4b)$$

Rods $$P_\theta = (1/u) \cdot \int_0^{2u} \left[(\sin w)/w\right] dw - \left[(\sin u)/u\right]^2 \qquad (4c)$$

where $x = \frac{kSD}{2}$, $y = \frac{k^2 S^2 R^2}{6}$, $u = \frac{kSL}{2}$

D = diameter of sphere

R = root-mean-square end-to-end distance of coil

L = length of rod

$K = 2\pi / \lambda^1$, $S = 2 \sin \theta/2$

λ^1 = actual wavelength in solution = $\frac{\lambda_o}{n_o}$

Evaluation of Experimental Data.

Since P_o for all models is unity, if the value of R_o can be established, the molecule weight can be determined. A reliable exterpolation to 0° can be made from measurements at several angles by the method of Zimm (1948). $\frac{Kc}{R_\theta}$ is plotted as a function of $\sin^2 \theta/2 + k^1 c$, where k^1 is an arbitrary constant chosen to spread the data suitably. A grid-like graph is obtained (see, for example Figure 17) and exterpolations along lines of constant angle and constant concentration are made to a common intercept upon the $\frac{Kc}{R_\theta}$ axis. This intercept $\left(\frac{Kc}{R_\theta}\right)_{\substack{c=0 \\ \theta=0}}$ is equal to the reciprocal of the weight-average molecular weight. Further useful information can be obtained from the limiting line $\left(\frac{Kc}{R_\theta}\right)_{c \to 0}$ of a Zimm plot (see Figure /

Figure 2). The ratio of the initial slope to the intercept defines, primarily, the radius of gyration, ρg,

$$\frac{\text{Initial Slope}}{\text{Intercept}} = \frac{16\,\Pi^2}{3}\, g^2 \left(\frac{n}{\lambda o}\right)^2$$

This can be related to the three models mentioned above by;

for a sphere $\rho g^2 = \frac{3D^2}{20}$

for a rod $\rho g^2 = \frac{L^2}{12}$

for a coil $\rho g^2 = \frac{r^2}{6}$

The dimensions determined thus, are the Z-average values and the ratio Mz/Mw must be known before the weight-average dimensions can be calculated. Benoit (1953) has elaborated the particle scattering factor theory to interpret data which show a curved $\frac{Kc}{R\theta}\ c \to o$ versus $\sin^2\theta/2 + k^1 c$ relationship.

In this case

$$\frac{\text{Slope of Asymptote}}{\text{Intercept of Asymptote}} = \frac{8\,\Pi^2}{3\lambda'}\, r_n^2$$

where r_n is the number-average radius of gyration.

Non-Ideal Behaviour.

So far only ideal solutions have been considered. However, at experimental concentrations, the molecules may no longer be so far apart that they can be considered as independent scatters. A certain amount of destructive interferences occurs causing a divergence from the linearity of the turbidity - concentration relationship /

relationship (equation)

$$\frac{Hc}{\tau} = \frac{1}{M} .$$

This can be accounted for by postulating a power series in c, thus;

$$\frac{Hc}{\tau} = \frac{1}{M} + 2Bc + 3Dc^2 + \ldots$$

The turbidity - concentration relationship then reassumes its linear character.

This postulate is justified since it arises naturally from the more rigorous approach to light-scattering theory by Einstein (1910) and Smoluchowski (1908, 1912) based on fluctuations in density throughout a liquid. It is shown that the constants B and D are constants involving the second and third virial coefficients, and they have the same significance as in osmotic pressure theory.

In most cases the term involving D is too small to be significant and the interaction constant, B, shows up as the slope of the zero angle line of the Zimm plot. Where the third virial coefficient term is significant a curve is obtained as predicted by theory (see, for example, Hyde and Taylor, 1963).

Large Values of the Relative Refractive Index.

The Rayleigh-Debye theory considers only small values of the relative refractive index (M). Mie (1908) has shown that if the field of radiation of a particle is represented by dipoles, quadrupoles, and poles of higher orders, with the centre of the particle as the origin, an exact and general solution of the scattering /

scattering can be obtained for isotropic transparent spheres which depends on the complex relative refractive index, the radius of the sphere, and the wavelength of the exciting radiation. However evaluation of this function is extremely complex and tables of these functions have been tabulated in only a few cases because the computation is so laborious.

It can be shown, however, that the Mie treatment yields both the Rayleigh equation (1) and the particle scattering factor for spheres (4a) for small relative refractive index values.

All substances measured by light-scattering in this thesis have low relative refractive index values.

THE LIGHT-SCATTERING PHOTOMETER.

The instrument used throughout this thesis was the Brice-Phoenix Light-Scattering Photometer Model 1000-D manufactured by the Phoenix Precision Instrument Company, Philadelphia, U.S.A. It is based on the design by Brice et al (1950) and a scheme of the optical system of the machine is illustrated in Figure 6 .

A full description of the photometer is not considered necessary since this is exhaustively dealt with by Brice et al (1950).

Experimental Technique.

With the cell in position and the photomultiplier set at 0^{o} with all the neutral filters in, the sensitivity is adjusted to give a high deflection. Readings are then taken with the photomultiplier set at the required angles and, high deflections are obtained by the withdrawal of suitable neutral filters. The experimental scattering ratio $\left(\frac{G\theta}{G_o}\right)$ is obtained from the experimental deflections (g_θ) by calculation of,

$$\frac{G\theta}{G_o} = \frac{g\theta}{g_o} \times \frac{F1234}{F_\theta}$$

where F_{1234} is the transmittance of the total neutral filter combination,

an F_θ is the transmittance of the neutral filter combination used at angle θ .

$\frac{G\theta}{G_o}$ is determined first for the solvent, then for a concentration series by the addition of aliquots of concentrated solution. $\left(\frac{G\theta}{G_o}\right)_{solvent}$ is then subtracted from $\left(\frac{G\theta}{G_o}\right)_{solution}$ to yield $\left(\frac{G\theta}{G_o}\right)_{corr.}$ values. This technique automatically corrects for any minor defects in /

in the cell geometry, and the clarification of the solvent.

$\left(\frac{G\theta}{G_o}\right)_{corr.}$ is related to the true Rayleigh Ratio either by relative calibration (see P. 20) or by the geometrical calibration, based upon the analysis of Brice and quoted by the manufacturers i.e.

$$R_{90} = k.\ (n^2\ \frac{Rw}{Rc})a.\ \frac{G90}{G_o}$$

values of k and the residual refraction correction $\left(\frac{Rw}{Rc}\right)$ are provided.

Clarification.

Light-scattering is obviously very dependent upon the use of dust-free solutions. (It can be shown that the intensity of scatter is proportional to the sixth power of the radius of the particle). Particles like dust (or microgel) scatter disproportionately to their concentration and also unsymmetrically, much more light being scattered in the forward direction.

Various techniques have been suggested - filtration through sintered glass filters (e.g. Jones (1959)), Elford membrane (Goring and Johnson (1952)), activated charcoal (Lochet (1950)(1951)) etc. or centrifugation notably by Schulz et al (1952) and Dandliker and Kraut (1956).

In the present work, it has been found that careful use of the various grades of Millipore filters (Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.) gave good results and were very convenient to use.

A /

A necessary accompaniment to clarification of the solutions is the elimination of dust from scattering cells and other glassware. This was achieved by suspending the vessels in a stream of condensing acetone vapour in an apparatus similar to that of Thurmond (1952). After removal from the apparatus the glassware was finally dried and cooled to room temperature by standing in a slow stream of filtered air.

Before the above treatment all glassware was thoroughly washed with detergent and rinsed with distilled water.

Calibration.

By careful analysis, Brice et al (1950, 1954) evaluated all the factors involved in an absolute geometrical calibration of the Brice-Phoenix Light Scattering Photometer. However this can be greatly affected by slight discrepancies in an individual machine from the ideal situation presumed by geometrical calibration. It is much more satisfactory, therefore, to calibrate individual machines by relative methods, which involve comparison of the experimental scatter with absolute turbidity determined spectrophotometrically.

The calibrating medium used must also fill several conditions:

(1) it must be small compared to the wavelength of the light used and it must have a correspondingly small dissymmetry.

(2) there must be no absorption in the wavelength range studied. This is shown by the linearity of the turbidity (τ) (or absorbance) as a function of the inverse of the fourth power of the wavelength (λ) or by the slope of $\log \tau$ versus $\log \lambda$ being close to 4, at any particular concentration.

(3) there must be negligible depolarisation of scattered light.

(4) it is preferable that the material should have a high scattering-power and that its solutionsshould be easily clarified and reproducible.

In recent years Ludox colloidal silica has been very often used for the absolute calibration, since it fits all of the above categories providing it is centrifuged at sufficiently high force-yield to remove aggregates before use. (Dezelic and Kratohvil (1960)). Other substances which have been shown to possess these characteristics are oil-free samples of Syton 2X (Jennings and Jerrard /

Jerrard (1964)) and Brewers Yeast Glycogen (Jones (1959)). Because of the fundamental nature of calibration, it was decided to calibrate with all three substances both from the point of view of increased accuracy and also in order to have some relative standard of comparison for the three substances.

EXPERIMENTAL.

Turbidities were measured in the 4 cm. matched silica cells both on the recording Perkin-Elmer 137 and on the Unicam SP 500 spectrophotometers. With care there was a less than 2% discrepancy between the two readings.

The scattering ratio was measured in the Brice-Phoenix Light-Scattering Photometer by measuring the ratio of the 90° scattered beam to the transmitted beam at 0°, in cylindrical cells type C-101.

The dissymmetries (I_{45}/I_{135}) were also measured and found to be close to unity. At high concentrations of Ludox they are smaller than unity, an effect that can be attributed to secondary scattering and the dilution effect (Elias (1958)).

Before use, stock solutions of Ludox and Syton 2X were centrifuged at ca. 20,000g. for 2 hours. All solutions were filtered through Millipore filters (Type RA, pore size 1.2) several times (ca.3) immediately before use.

Because of the greater reproducibility noted when Ludox was diluted with 0.05M sodium chloride rather than water (see e.g. Goring et al (1957)), this solvent was used to dilute both the Ludox and the Syton 2X. The Brewers Yeast Glycogen was diluted with water.

Relation of Turbidity to 90° Scattering Intensity. The method used is a variation of that proposed by Maron and Lou (1954).

The excess turbidity (τ) of a solution due to small and isotropic solute particles is given by

$$\tau = \frac{16\,\Pi\,r^2}{3V}\;\frac{I_{90}'}{I_0'}$$

where V = scattering volume

r = distance between V and point of measurement

I_{90}' = intensity of light scattered at 90° at V

I_0' = average intensity of light incident upon V

It can be seen from this equation that τ/I_{90}' is constant at constant incident light intensity for any one wavelength, and this relationship has been used for the calculation of calibration constants. (See, for example, Oster (1950)). However the actual experimental quantities measured differ from I_{90}' and I_0' by amounts determined by the Lambert-Beer relation. If the experimental values measured are I_{90} and I_0 then

$$I_{90} = I_{90}'\, e^{-\tau X_1}$$

$$\text{and } I_0 = I_0'\, e^{-\tau X_2}$$

where X_1 and X_2 are the distances travelled by the beam, through solution, from V.

$$\text{Therefore } \tau = \frac{16\,\Pi\,r^2}{3V}\;\frac{I_{90}\, e^{\tau X_1}}{I_0\, e^{\tau X_2}}$$

$$\therefore \frac{\tau}{I_{90}} = \frac{16\,\Pi\,r^2}{3V\,I_0}\; e^{\tau(X_1 - X_2)}$$

$$\therefore \log_{10}\frac{\tau}{I_{90}/I_0} = \log_{10}\frac{16\,\Pi\,r^2}{3V} + \left(\frac{X_1 - X_2}{2.303}\right)\tau$$

$$\text{OR } \log\frac{\tau}{I_{90}/I_0} = k_1 + k_2\tau$$

where k_1 and k_2 are constants.

constants.

Hence plotting log (τ/G'_{90}) versus τ should result in a straight line $(G'_{90} = \frac{G_{90}}{G_0})$. If $\tau \to 0$, which is the case in all exterpolations to zero concentration, log (τ/G'_{90}) remains constant and $(\tau/G'_{90})_0 = C$, the calibration constant.

RESULTS.

Transmission Measurements. Figures 3 and 4 show the variation of turbidity with wavelength over the range 4000-6000Å for different concentrations of the three calibrating media. It can be seen that graphs of Ludox and Brewers' Yeast Glycogen (BYG) are completely linear at all concentrations over the range studied. Syton 2X, however, exhibits distinct divergence from linearity at low wavelengths and high concentrations. However it is completely linear at 5461Å over all the concentrations and appreciable variation only occurs in the higher concentration ranges at 4358Å. Consequently only the more dilute concentrations were used for calibration purposes.

Scattering Measurements were made in the manner described above.

Calculation of Calibration Constant, C. Figures 5a and 5b show the experimental data plotted according to the derived equation. It can be seen that, within experimental error,

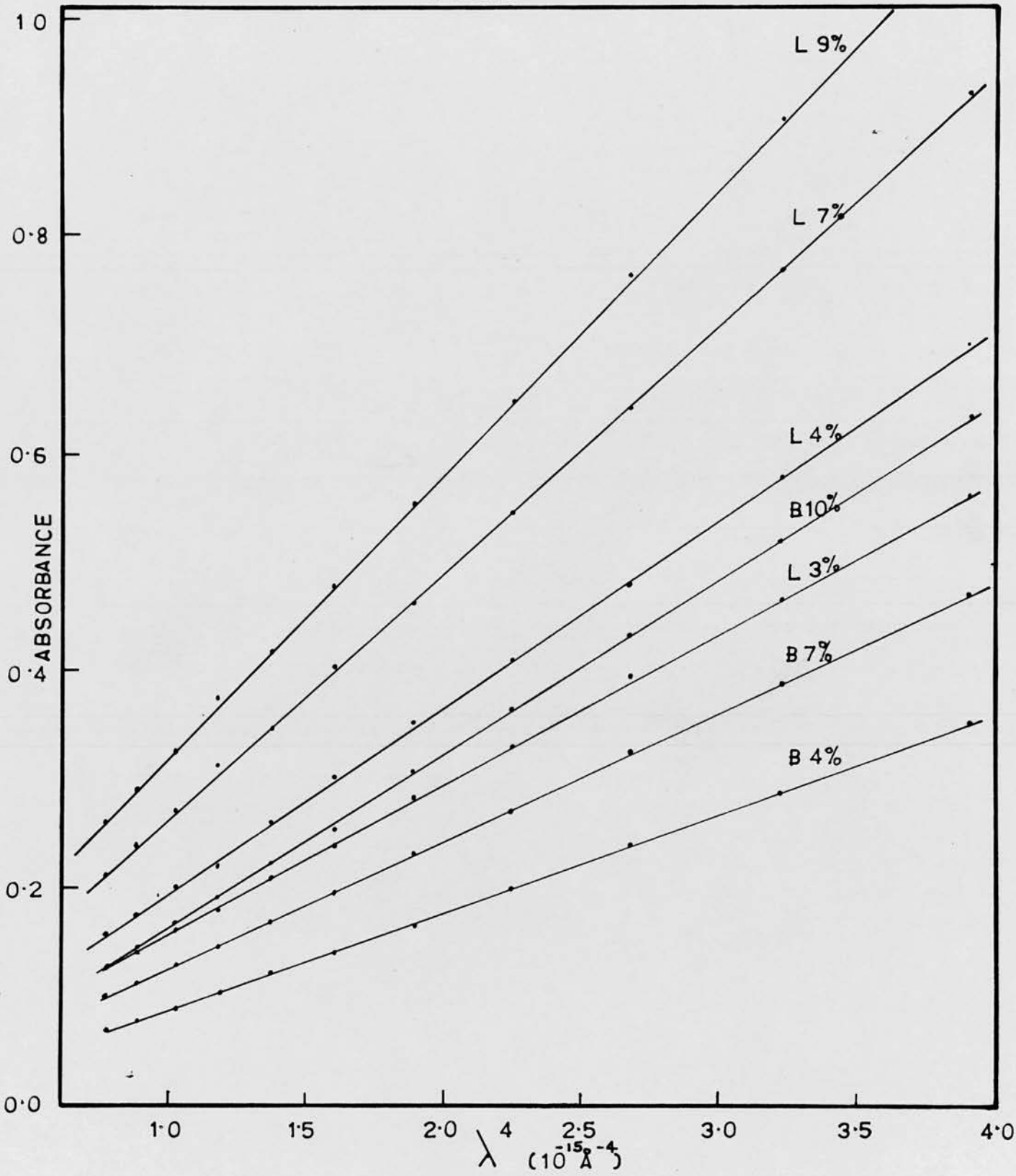
(1) the points are best fitted by straight lines

(2) at any one wavelength the exterpolations to $\tau = 0$ are coincident.

The value of the calibration constants calculated from these results are

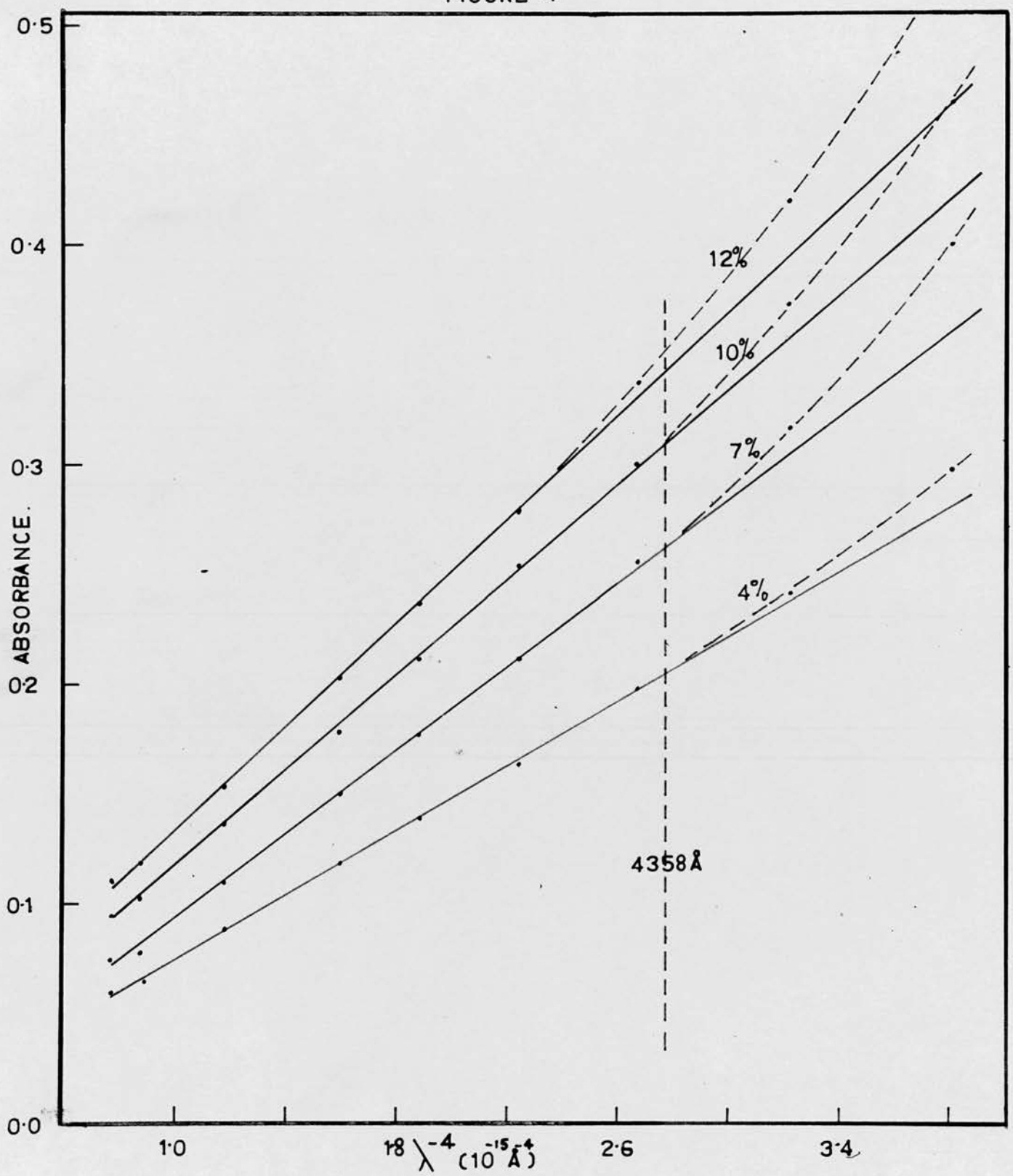
λ	C
5461Å	0.103 ± 0.003
4358Å	0.085 ± 0.002

FIGURE 3



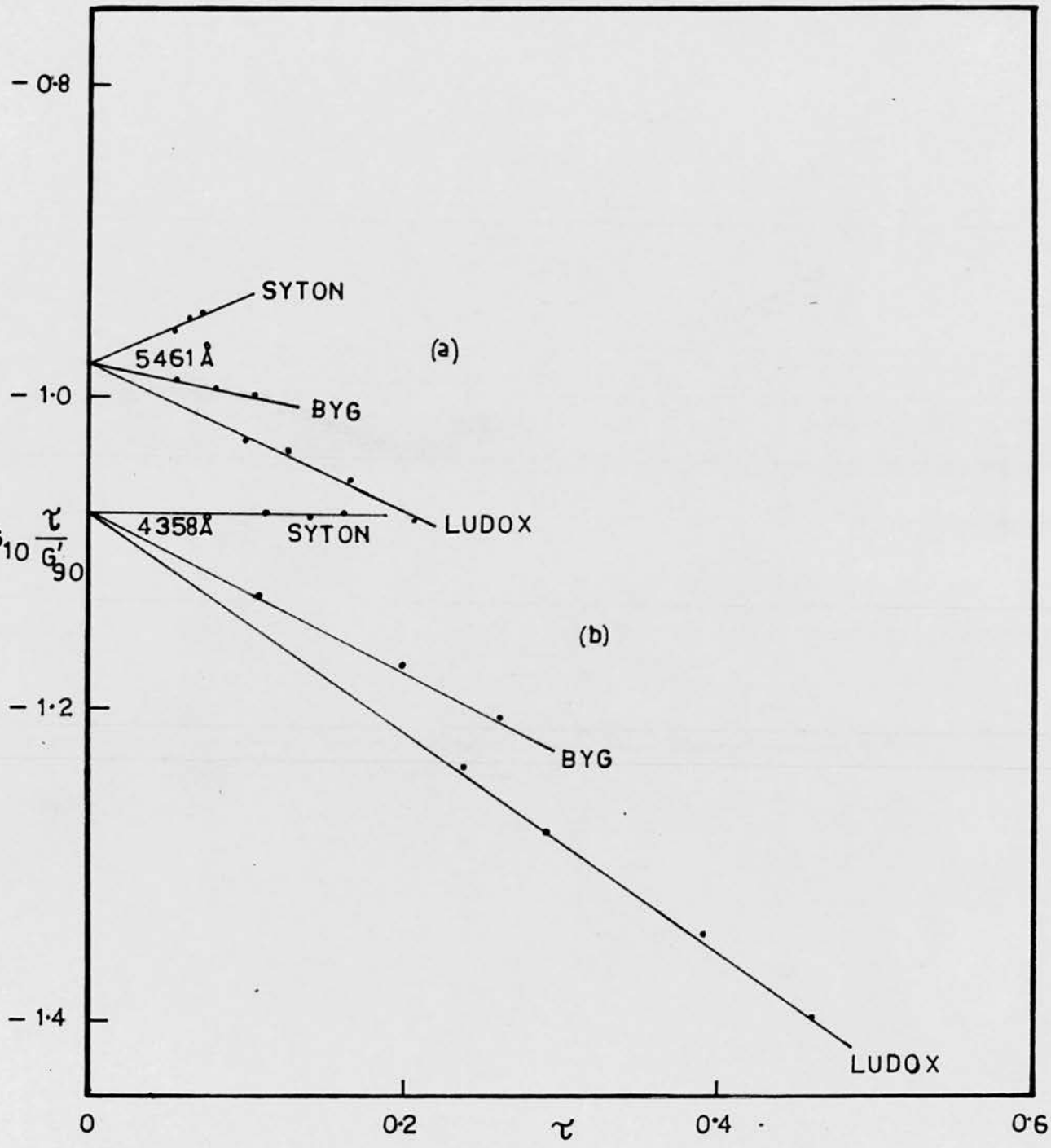
LUDOX (L) and BYG (B) — TRANSMISSION MEASUREMENTS.

FIGURE 4



SYTON 2X — TRANSMISSION MEASUREMENTS.

FIGURE 5



CALIBRATION DATA.

Using these values experimental values for the R_{90} value of benzene were found to be within 3% of those reported by Kratohvil et al (1962).

CONCLUSION.

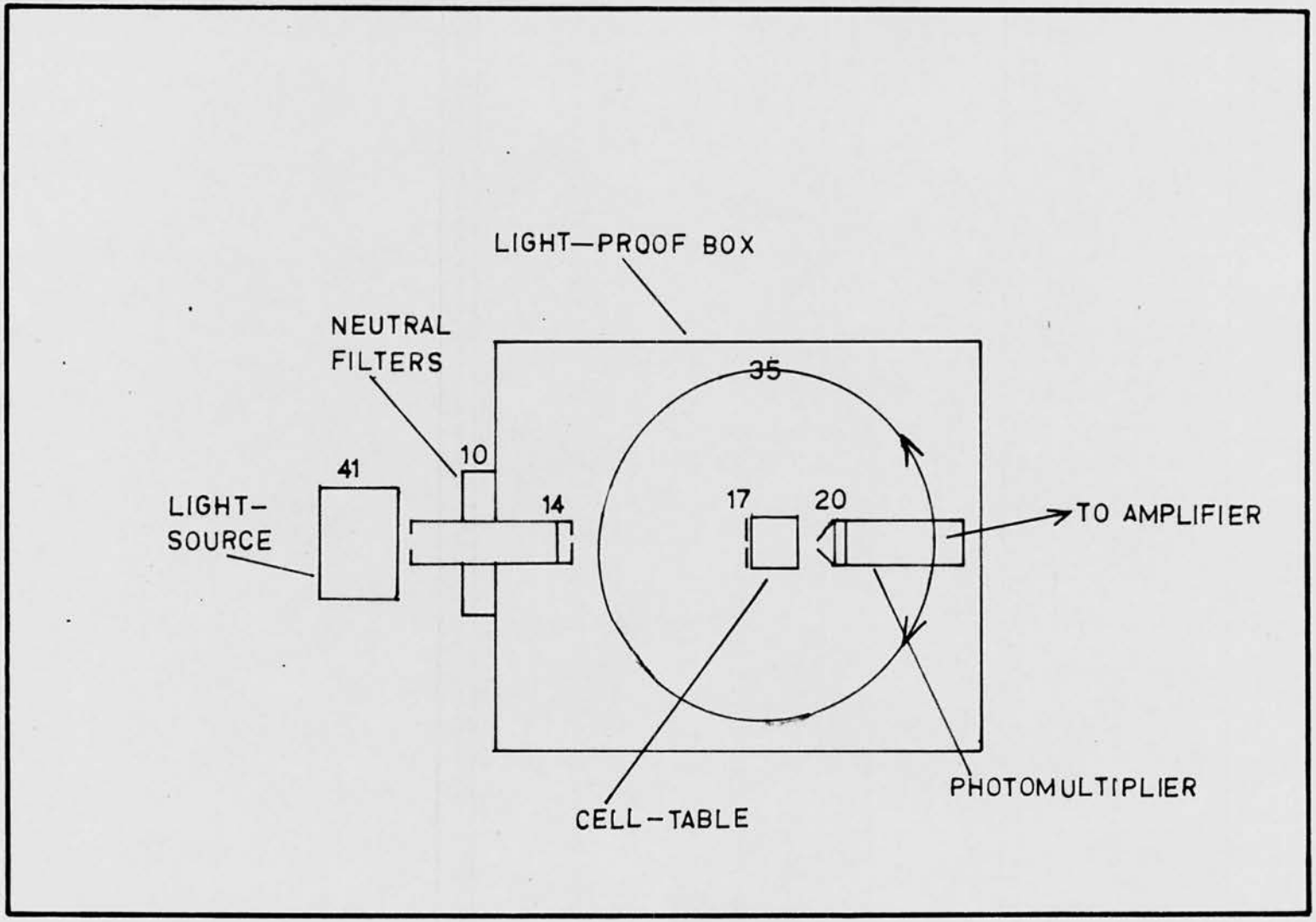
All three calibration media used were found to be suitable for the purpose providing that

(1) the Ludox or Syton solutions were centrifuged before use to remove aggregates

(2) the Syton solutions were sufficiently dilute.

Ludox solutions are to be preferred for calibration purposes because of their conveniently wide range of turbidities, as compared to Syton 2X, which enhances the accuracy of the exterpolation and because of their availability in commercial form, as compared to Brewers' Yeast Glycogen which must be carefully extracted (Jones (1959)).

FIGURE 6



SCHEME of LIGHT—SCATTERING PHOTOMETER.

Light-Scattering (Experimental)

As the technique of light-scattering is very dependent for its absolute accuracy upon several instrumental constants, these have been critically evaluated. The method of alignment advocated by the manufacturers (Brice-Phoenix Light-Scattering Photometer Operation Manual, OM-1000; Phoenix Precision Instrument Company, U.S.A.) has been found to be unsatisfactory and has been replaced by the following technique.

Realignment of Optical System.

(Items are numbered as in the instruction manual. See also the schematic diagram (Fig. 6)).

(1) Remove photomultiplier nosepiece (Item 20) and plug the opening from the rear with a small cork, then replace nosepiece. The nosepiece must be plugged to protect the photomultiplier tube from the intense light used when checking optical alignment.

Insert narrow diaphragm system.

(2) Remove all neutral filters from the beam by pulling all the push-rods (Item 10) to the out position.

Remove the working standard and the standard opal.

Index the monochromatic filter turret to an empty opening (white light).

Observe the light beam image at diaphragm 17 by means of an angled mirror. The beam should be centred vertically and horizontally.

(3) If the beam is not centred vertically, this can be corrected by removing the mercury-vapour lamp-housing (screws 41) and adjusting the lamp height by means of the Allan screw (Item 38).

(4) If the beam is not centred laterally the following procedure should be adopted.

/adopted.

(a) A clean, semi-octagonal cell (Cat. no. D-101) is filled with fluoroscein solution and placed on the cell table. The back-reflection observed on the outside of diaphragm 14 is made symmetrical about the incident beam by loosening the locking screws (Item 6) and moving the lamp-housing laterally. The lamp-housing is then locked in position.

(b) The beam hitting the cell table diaphragm (17) is then centred laterally by loosening the Allan screw (Item 19) and rotating the table.

(c) The disc (Item 35) is then rotated to the 0^{o} position. If diaphragm 20 is not symmetrically in the beam this can be corrected by placing it symmetrically and adjusting the zero by loosening the locking-screw (Item 32).

(5) Close shutter.
Index monochromatic turret to a filter position.
Remove cork plug from nose-piece.

Angular Uniformity.

In order to confirm the symmetry of the apparatus the distribution of fluorescent light intensity in a large cylindrical cell (Cat. no. C-101) was measured. Since fluorescence is radiated equally in three dimensions, the $1/1 + \cos^2\theta$ is not used and the measured intensities are only multiplied by $\sin\theta$ (to account for the increase in scattering volume "seen" by /

by the photomultiplier at angles other than 90°). Typical results for two cylindrical cells are shown in Table 1. It can be seen that very good constancy had been achieved (within 1.5%). The fluoroscein solution was that recommended by Goring (1953) i.e. 1mgm. per litre of fluoroscein in 0.1M sodium acetate solution.

TABLE 1.

Angular scattering of fluoroscein in two cylindrical cells.

CELL 1			CELL 2	
G	G sin θ	θ	G sin θ	G
90.7	45.4	30	47.1	94.2
71.0	45.6	40	47.5	74.0
60.0	46.0	50	47.7	62.3
53.0	45.9	60	47.8	56.2
49.0	46.0	70	47.9	51.0
46.8	46.1	80	48.0	48.7
46.0	46.0	90	47.9	47.9
46.8	46.1	100	47.9	48.6
48.9	45.9	110	47.8	50.9
52.9	45.8	120	47.6	55.0
59.6	45.7	130	47.5	62.0
64.3	45.5	135	47.4	67.0

Transmittance of neutral filters.

In the Brice-Phoenix instrument the difference between the scattered and transmitted light intensities are equalised by the insertion of neutral filters of known transmittance into the incident beam. Tomimatsu and Palmer (1959, 1961) have stated that the transmittance of the neutral filters as determined by the product of the individual filter transmittances (the procedure recommended by the manufacturers) differs significantly (up to 7%) from the experimentally determined transmittance of the particular filter combination. This was checked by experimentally determining the transmittances of the individual filters and of all the possible filter combinations (See Brice-Phoenix Manual). The transmittances were determined for both the blue (436mμ) and the green (546mμ) line and each experimentally determined value was the mean of ten individual readings. The results (Table 2) do not show the percentage discrepancy indicated by Tomimatsu and Palmer.

TABLE 2.

Neutral Filter Transmittances.

Transmittance (Experimental)	546mμ Transmittance (Calculated*)	% Discrepancy	Filter Combination ≠	% Discrepancy	436mμ Transmittance (Calculated*)	Transmittance (Experimental)
0.4857	-	-	1	-	-	0.4618
0.2873	-	-	2	-	-	0.2595
0.1330	-	-	3	-	-	0.1108
$0.5321_{x10^{-1}}$	-	-	4	-	-	$0.4714_{x10^{-1}}$
0.1392	0.1395	0.22	12	1.00	0.1208	0.1196
$0.6450_{x10^{-1}}$	$0.6460_{x10^{-1}}$	0.15	13	0.25	0.5117_{1} $_{x10^{-1}}$	$0.5130_{x10^{-1}}$
$0.2588_{x10^{-1}}$	$0.2584_{x10^{-1}}$	0.15	14	0.32	0.2177_{1} $_{x10^{-1}}$	$0.2184_{x10^{-1}}$
$0.3816_{x10^{-1}}$	$0.3821_{x10^{-1}}$	0.13	23	0.21	0.2876_{1} $_{x10^{-1}}$	$0.2882_{x10^{-1}}$
$0.1519_{x10^{-1}}$	$0.1523_{x10^{-1}}$	0.66	24	0.25	0.1223_{1} $_{x10^{-1}}$	$0.1226_{x10^{-1}}$
$0.7069_{x10^{-2}}$	0.7077_{2} $_{x10^{-2}}$	0.11	34	0.36	0.5223_{2} $_{x10^{-2}}$	$0.5242_{x10^{-2}}$
$0.1853_{x10^{-1}}$	0.1855_{1} $_{x10^{-1}}$	0.11	123	0.30	0.1339_{1} $_{x10^{-1}}$	$0.1335_{x10^{-1}}$
$0.7403_{x10^{-2}}$	0.7410_{2} $_{x10^{-2}}$	0.10	124	0.32	0.5655_{2} $_{x10^{-2}}$	$0.5672_{x10^{-2}}$
$0.3439_{x10^{-2}}$	0.3437_{2} $_{x10^{-2}}$	0.06	134	0.37	0.2412_{2} $_{x10^{-2}}$	$0.2421_{x10^{-2}}$
$0.2025_{x10^{-2}}$	0.2033_{2} $_{x10^{-2}}$	0.39	234	0.22	0.1356_{2} $_{x10^{-2}}$	$0.1353_{x10^{-2}}$
$0.9889_{x10^{-3}}$	0.9871_{3} $_{x10^{-3}}$	0.18	1234	0.84	0.6312_{3} $_{x10^{-3}}$	$0.6259_{x10^{-3}}$

* Calculated from the products of the transmittances of the individual filters.

≠ Filters numbered as in the Brice-Phoenix Light-Scattering Photometer Operation Manual, OM-1000

II. b. VISCOSITY.

Perhaps the most characteristic property of a polymer is the increase in relative viscosity it causes in any medium in which it is dissolved. The applied force on the polymer molecule is the flow of the solvent under gravity, and the actual viscosity measured is the contribution to the viscosity of the solution made by the polymer molecules.

The ratio of the viscosity, η , of a solution to the viscosity, η_o, of the pure solvent is called the relative viscosity η_r

$$\eta_r = \eta / \eta_o$$

The relative increase in the viscosity is called the specific viscosity, η_{sp}

$$\eta_{sp} = \frac{\eta - \eta_o}{\eta_o} = \eta_r - 1$$

The viscosity number, η_c, is the intrinsic viscosity divided by the concentration,

$$\eta_c = \eta_{sp}/c$$

In order that the viscosity may be uncomplicated by non-Newtonian behaviour, the viscosity number is exterpolated to infinite dilution to yield the limiting viscosity number or intrinsic viscosity ($[\eta]$).

$$[\eta] = \lim_{c \to o} \eta_c = \lim_{c \to o} \eta_{sp}/c$$

The units of c used here are g./ml. as recommended by the I.U.P.A.C. (1952).

Viscosity /

Experimental.

The viscometers used in this work were of a modified Ubbelohde type (Ubbelohde (1937), Davies and Elliot (1949)). In a capilliary viscometer of this type the viscosity is given by

$$\eta = K\rho t - \frac{B\rho}{t}$$

where K and B are constants

t is the flow time of a fixed volume

and ρ is the density of the liquid.

B, the kinetic energy factor, arises because the liquid leaves the capilliary with finite velocity. By the use of standard liquid (Hall and Fuoss (1951)), the value of B for the viscometers used in this work, has been shown to be negligible (Banks (1960)).

Hence $\eta = K\rho t$ for the solution

and $\eta_o = K\rho_o t_o$ for the solvent.

The relative viscosity is therefore given by,

$$\eta_r = \frac{K\rho t}{K\rho_o t_o}$$

and the specific viscosity by,

$$\eta_{sp} = \frac{\rho t - \rho_o t_o}{\rho_o t_o}$$

For dilute polymer solutions $\rho = \rho_o$ to a close approximation, hence,

$$\eta_{sp} = \frac{t - t_o}{t_o}$$

Having thus obtained experimental values of η_{sp} for several concentrations a graph of $\eta_{sp/c}$ against c was drawn. By extrapolation of the plot to infinite dilution the intrinsic viscosity, $[\eta]$, was obtained.

Procedure.

Both solvent and solution were filtered through G4 sintered glass filters before use. The viscometers were immersed in a thermostated bath (25 ± 0.01°C). The flow times were measured to the nearest 0.1 second by means of a stop-watch.

A measured volume of filtered solution was initially added to the viscometer. Additions of aliquots of pure solvent provided the dilution series required for the exterpolation to zero concentration. The solvent flow time was determined on a separate run.

II.c. ULTRACENTRIFUGATION.

Colloidal particles in fluid, under sufficiently high force-fields, reach a terminal velocity whose value is determined by the frictional properties of the particles and the strength of the field. Ultracentrifuges capable of producing such fields have been designed by Svedberg and others (Svedberg and Pedersen, (1940)).

Svedberg defined the sedimentation coefficient (S) as the velocity of sedimentation under unit centrifugal force

i.e. $S = (dx/dt)/w^2x$ c.g.s. units

$= (dx/dt)/w^2x \times 10^{13}$ Svedberg units

where x = distance from centre of rotation (cms.)

w = angular velocity (radians/sec.)

The theory of ultracentrifugation is well established and full details of it may be found in Svedberg and Pedersen (1940) and Schachman (1959).

EXPERIMENTAL /

EXPERIMENTAL. A Spinco Model E Analytical Ultracentrifuge was used throughout this work, full details of which may be found in the relevant handbook. A Rotor Temperature Indicating and Control Unit was available, by means of which the rotor temperature could be controlled to $\pm$ 0.02°C during sedimentation. Boundaries were observed and recorded photographically by the Schlieren optical system.

II.d. CONDUCTIVITY.

The specific conductance is defined as the inverse of the specific resistance of an electrolyte solution. (See, for example, Robinson and Stokes (1959), where full details of conductivity theory may be found).

Conductivity measurements of colloidal solutions have been mainly concerned with detergent solutions. (See, for example, Tarter and Wright (1939)). For studies of this type it has been shown (Alexander and Johnson (1949)) that aggregation of charged colloids has three main consequences on the conductivity:

(1) viscous drag is reduced.

(2) the ionic atmospheres of oppositely charged gegenions exert a much increased "braking" effect.

(3) some gegenions will adhere to the charged colloids due to their high charge and thus will be forced to travel in the opposite direction to that of free gegenions.

Under normal conditions (2) and (3) will outweigh (1) resulting in a slower increase in specific conductivity with increasing concentration /

concentration for the colloidal aggregates as compared to the free colloid.

EXPERIMENTAL.

A standard Lock Conductivity Bridge (Type BC1) was used with the commercially available cell.

Conductivity water was obtained by passing stone-distilled water through an "Elgastat" ion-exchange column.

All measurements were made in sealed polythene tubes immersed in a thermostated ($\pm$0.05°C) water bath.

II.e. ESTIMATION OF POLYSACCHARIDE CONCENTRATION.

The polysaccharide was converted by acid hydrolysis, to free glucose which was estimated by alkaline ferricyanide (Lampitt et al (1956).

SECTION III.

STUDIES ON AMYLOPECTIN.

SECTION III

STUDIES ON AMYLOPECTIN.

INTRODUCTION.

The starch granule is insoluble in cold water but upon heating the granules can be disrupted to form a "dispersion". This dispersion contains two chemically distinct species; amylose and amylopectin. These can be readily separated by the addition of a polar, organic molecule, when amylose forms an insoluble-complex (Schoch (1945)). The amylopectin is obtained from the supernatant by freeze-drying. In general, starch granules contain substantially more (ca. 80%) of the amylopectin than the amylose component. The nature of the complex biosynthesis which must be involved in the formation of such a granule is discussed in Section IV. Amylose is discussed in Section VI.

The amylopectin components isolated from aqueous dispersions of starches from many sources have been characterised (Greenwood and Thomson, (1962)). The average chain-lengths varied between ca. 20 and ca. 28 glucose units depending upon the source. While the β-amylolysis limit (see p. 83) remained effectively constant (56-59%), there were large differences in the phosphorus contents of the samples (0.02% - 0.1%).

In 1935, Posternak showed that most of the phosphorus in cereal starches was in the form of phosphatides which could be extracted by hot water or methanol. On the other hand, the phosphorus in tuber starches, such as potato, sago, tapioca etc. was found to be linked to the polysaccharides in ester form through the /

the C-6 position. Most of the phosphorus in the starch (ca. 90%) has been shown to be linked to the amylopectin molecules (Schoch) (1942). Fukui (1958) has investigated the effect of phosphatase upon the esterified phosphate groups in potato starch. It was found that although the phospho-monosaccharide obtained from the acid-hydrolysate of the starch was attacked by the enzyme at the same rate as glucose-6-phosphate, the attack on the phosphate groups in amylopectin or lower molecular-weight dextrins was considerably impeded. It was suggested that this could be due to interaction of neighbouring glycosyl chains with the phosphate group. These results have been confirmed by MacGregor (1964).

Amylopectins from various sources have been shown to have consistently high molecular weights (see Greenwood (1956)), e.g. 500×10^6 for a sample of potato amylopectin (Greenwood (1960)), but it has been suggested (Erlander and French (1958)) that the large amylopectin molecules obtained from corn endosperm may be chemical aggregates of four.

EXPERIMENTAL.

Isolation and Purification of Amylopectins: The starch obtained from potato was isolated and purified as described by Greenwood and Thomson (1958) and then pretreated with liquid ammonia (Banks et al (1959)). After dispersion to give a 0.5% solution at 98° under nitrogen the amylose was removed as the thymol-complex by high-speed centrifugation. The amylopectin was obtained by freeze-drying of the supernatant. The purity of all the samples used in the present work, as shown by iodine-affinity, was > 99%

Titration /

Titration Measurements on the amylopectins were made with the Radiometer (Copenhagen) Recording Titrimeter.

All other techniques are as detailed in Section II.

RESULTS.

Preparation of "Amylopectin-Acid": As isolated directly from the tuber, the acidic glycogens of the phosphate groups bound to amylopectin are replaced by metal ions. (Nutting (1952)). In order that the behaviour of the amylopectins might be unaffected by these ions, they were removed by passing the amylopectin solutions through an ion-exchange column. The efficiency of the exchange was followed by measuring the potassium and sodium contents of amylopectin solutions (ca. 0.05%) by flame-photometry. (The amylopectins were hydrolysed by hydrobromic acid and the machine was calibrated with standards).

The results show that a substantial reduction is made in the sodium content of the amylopectins. The pH of the column effluent was ca.3.5 as compared with the original pH of ca. 6.5. Dialysis has no effect upon the metal ion content of the amylopectin.

AMYLOPECTIN

	Original	Dialysed	Ion-Exchange
Na (p.p.m.)	1.5	1.3	0.2
K (p.p.m.)	0.4	0.4	0.1

The ion-exchange column was Duolite C-63 (or Biorex c-63) a phosphoric acid ion-exchanger. Substantially the same results were obtained with Amberlite IR-120, but it was thought that there might be some possibility of degradation of the amylopectin on such a strongly acidic column. Amylopectin substantially converted to its free acid form as above, will be called "amylopectin-acid".

Titration of Amylopectin-Acid: In order to confirm that the phosphorus in amylopectin was in the form of orthophosphate, a sample of amylopectin-acid (Phosphorus content = 0.05%) was titrated against CO_2-free sodium hydroxide under an atmosphere of nitrogen. The titration curve for this (the average of ten separate runs) is shown in figure 7 , as is the curve for orthophosphoric acid at approximately the same concentration of phosphorus as in the amylopectin.

The results show, in agreement with the work of Briggs and Hanig (1946) on electrodialysed starches, that there is fair agreement between the two samples:-

	pK_1	pK_2
Amylopectin Acid	2.0	7.2
Orthophosphoric Acid	1.96	7.13

(Results evaluated by the method of Van Slyke (1922)).

This confirms the existence of phosphate groups in potato amylopectin.

Expansion of Amylopectin-Acid: The existence of ionisable groups in amylopectin allows the polysaccharide to have a negative charge. Repulsion of the negative phosphate groups will cause the polysaccharide molecule to expand. If, however, the amylopectin is dissolved in an electrolyte solution, such as sodium chloride, the expansion of the molecule will depend on the strength of the surrounding ionic atmosphere. This has been confirmed from the light-scattering measurements. In figure 8 , the zero-concentration lines of Zimm-plots for amylopectin acid measured in various molarities of saline, water, and 0.1 M sodium hydroxide solution are shown. /

shown. The radii of gyration calculated from these data are shown below:

Solvent	ρ_z (Å)	Volume* (Å^3 x 10^{-8})	% Volume Expansion ≠
10^{-1}M NaCl	2090	708	-
10^{-3}M NaCl	2360	1010	44
10^{-1}M NaOH	2470	1160	65
H_2O	2540	1260	80
10^{-4}M NaCl	2610	1370	95
10^{-5}M NaCl	2760	1620	131

* Based on simple sphere

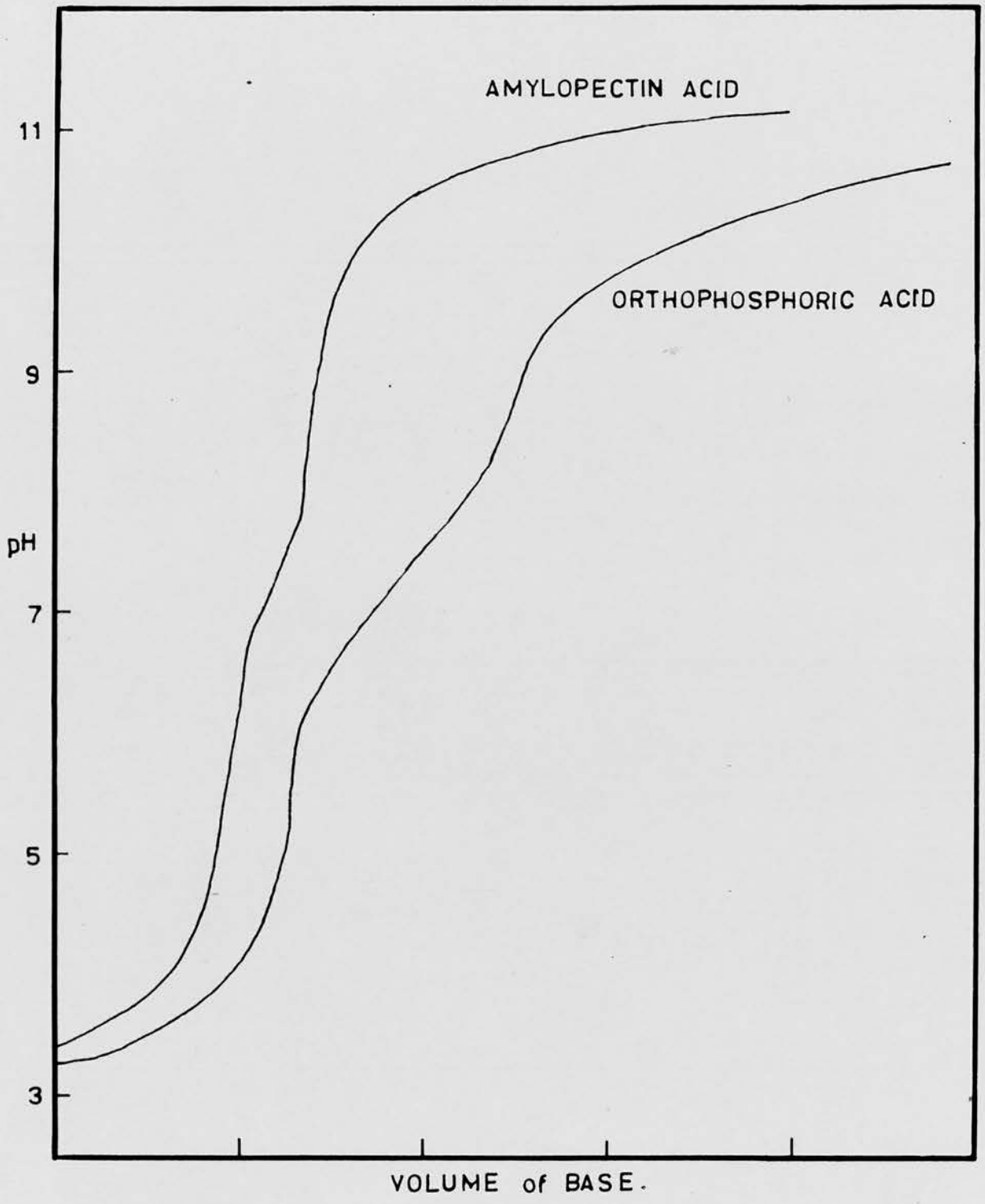
≠ Relative to volume in 10^{-1}M NaCl

By using a sphere as a very simple approximation to the shape of the amylopectin molecule in solution, a rough idea of the magnitude of the volume changes involved may be gained.

No evidence was found for the increase in molecular weight, and particle radius as compared to amylopectin in 10^{-5}M saline, which was reported for potato amylopectin by Witnauer, Senti, and Stern (1955) at salt concentrations of 10^{-3}M. Instead a smooth gradation, at constant molecular weight, is found from an open to a denser structure, as shown by calculations of the particle-scattering factor (figure 9).

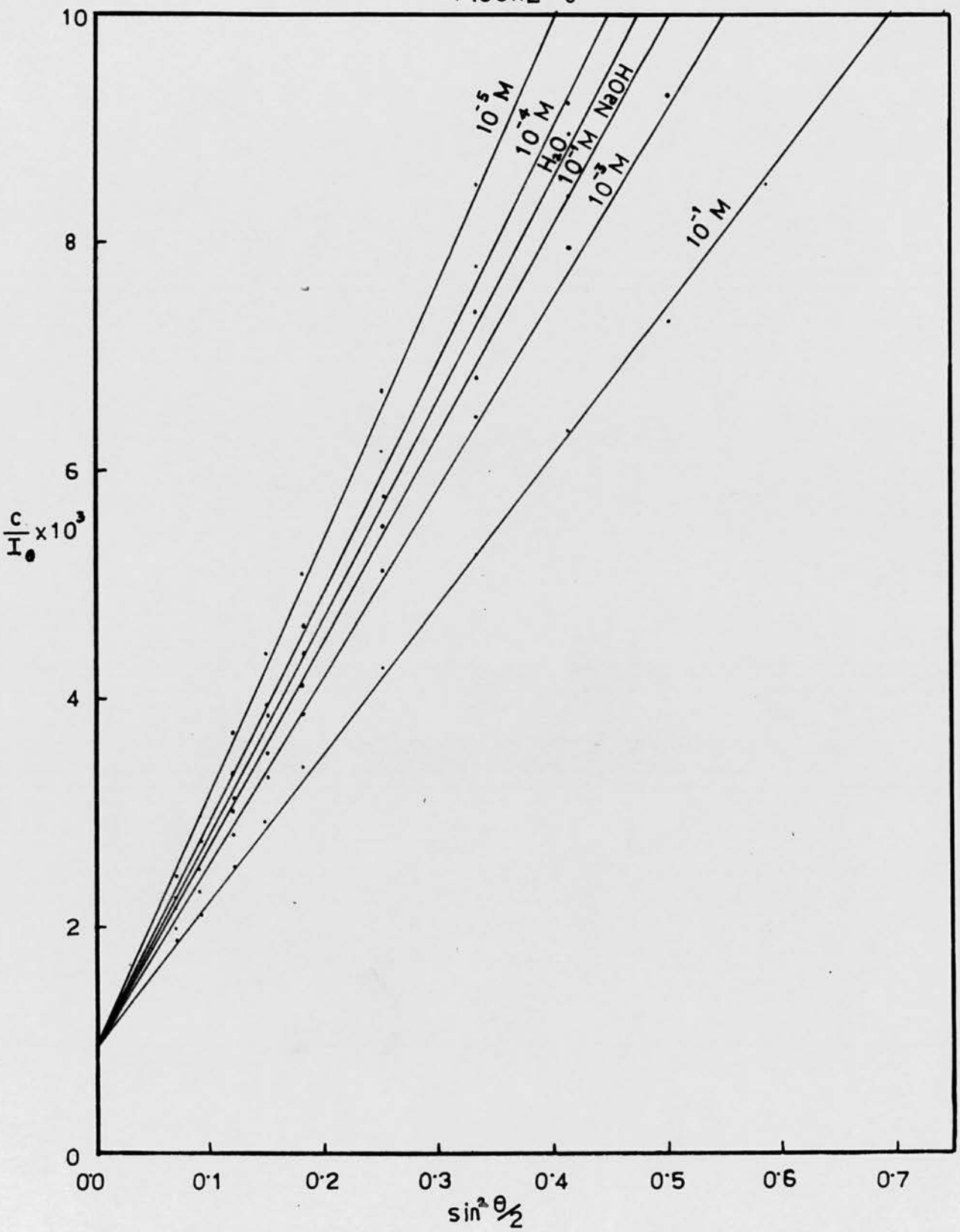
When these corresponding configurational changes are followed by viscosity, curves like these illustrated in figure 10 are obtained. These show the maxima at low concentration of saline and high dilution typical of polyelectrolytes. (Viscosity measurements in this very dilute region were achieved by the construction of viscometers /

FIGURE 7



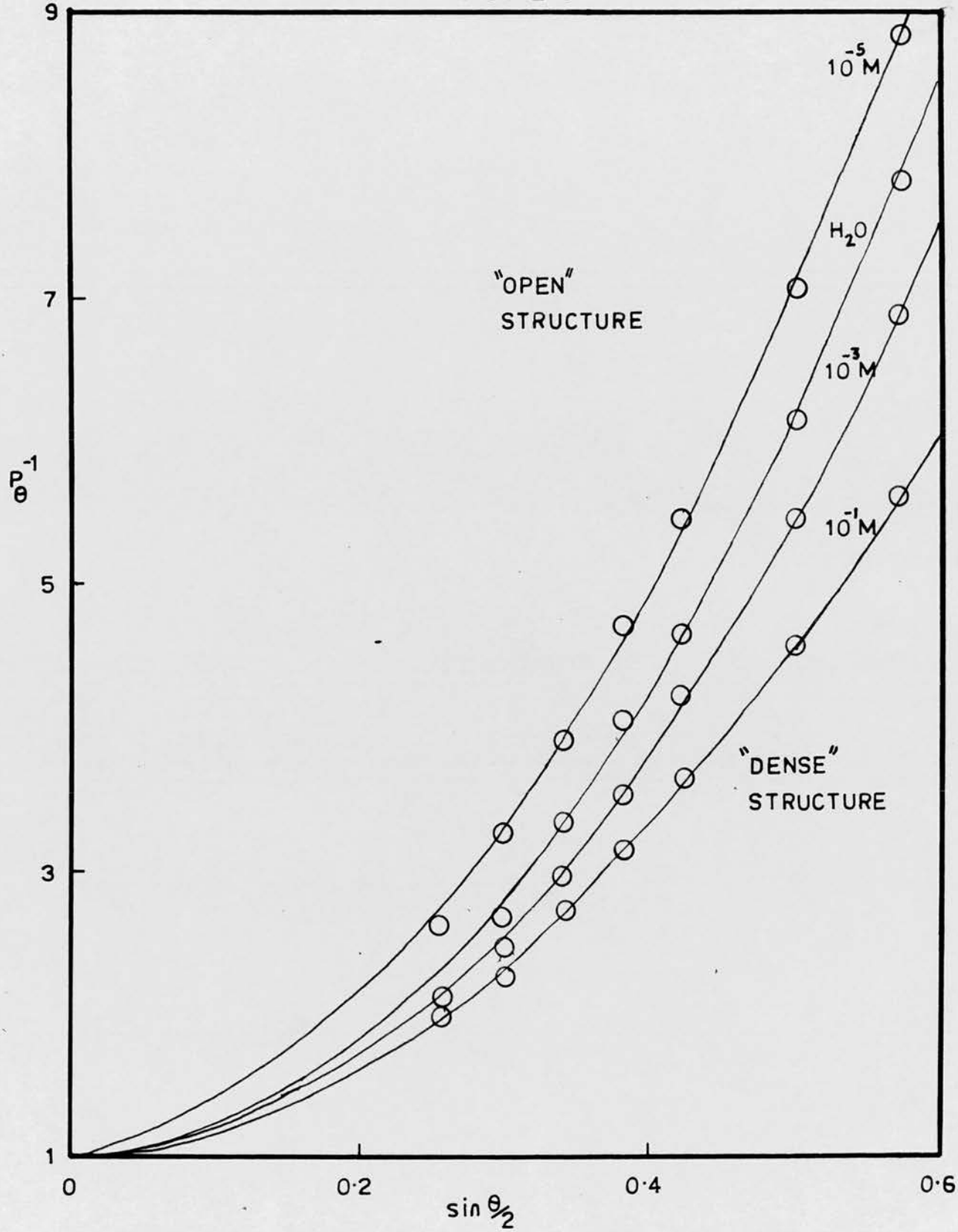
TITRATION CURVES.

FIGURE 8



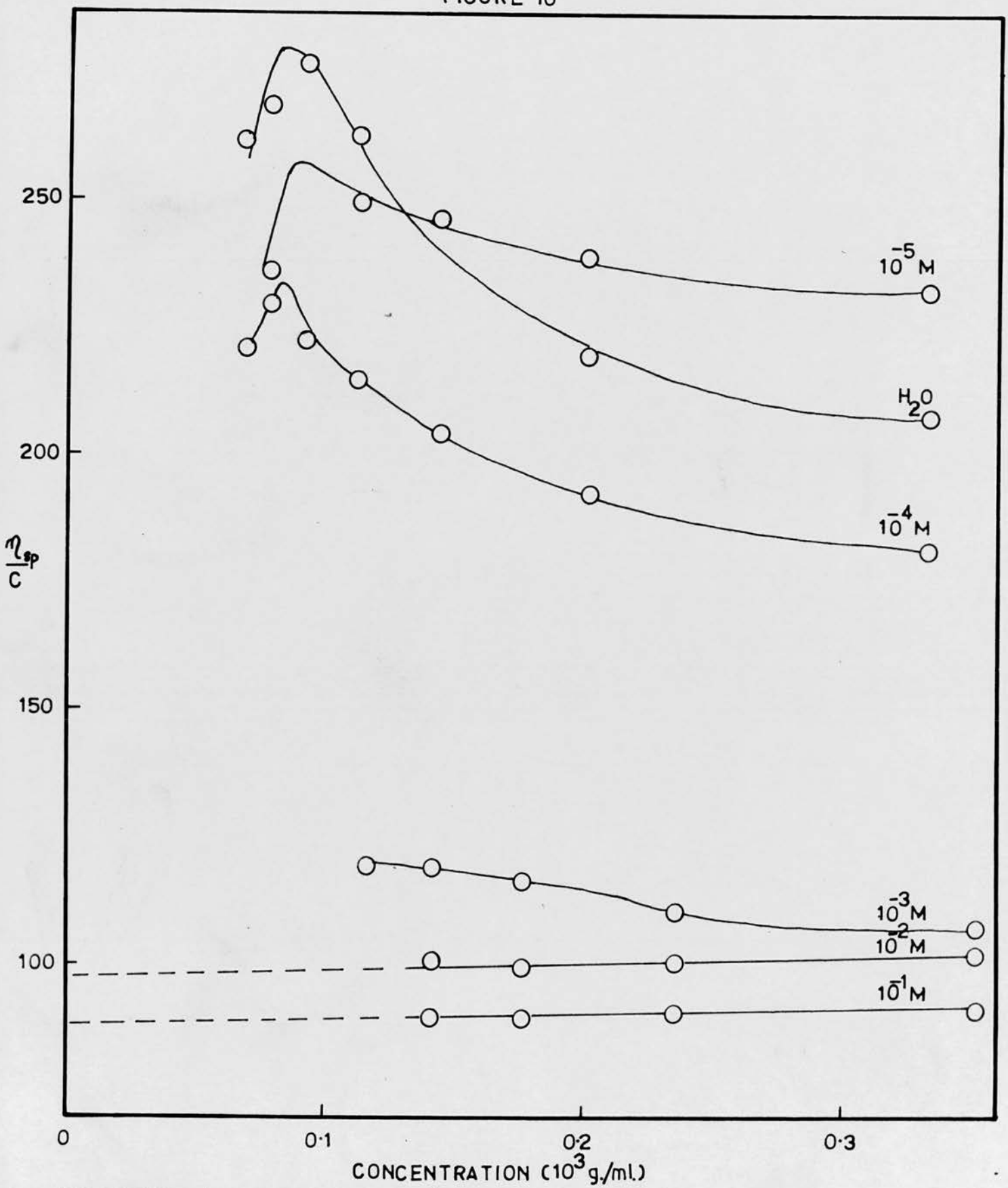
VARIATION of RADIUS of GYRATION of AMYLOPECTIN ACID with SALT MOLARITY.

FIGURE 9



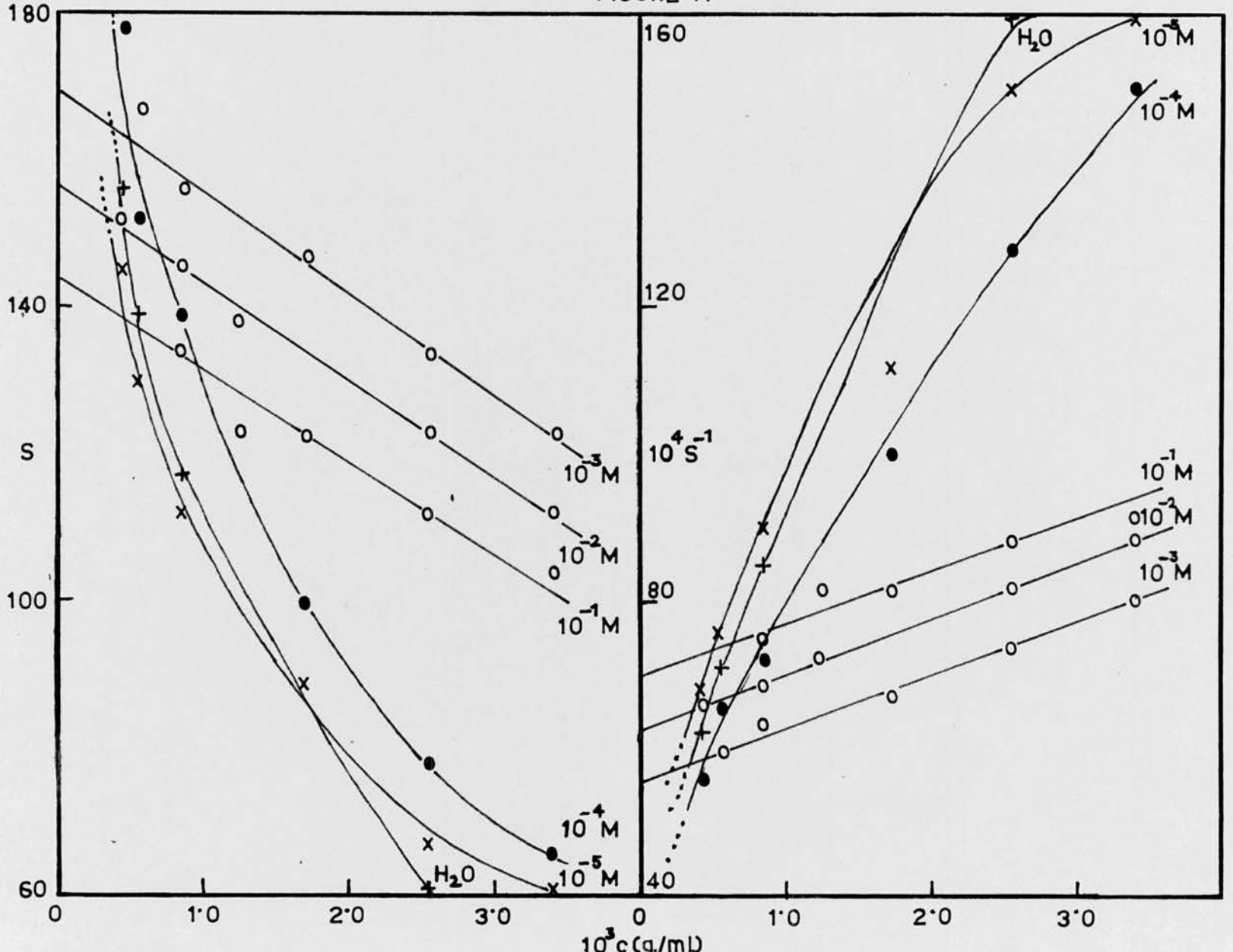
VARIATION of SHAPE of AMYLOPECTIN ACID with SALT MOLARITY.

FIGURE 10



VISCOSITY of AMYLOPECTIN ACID in various MOLARITIES of SALT.

FIGURE 11



SEDIMENTATION BEHAVIOUR of AMYLOPECTIN ACID in various MOLARITIES of SALT.

viscometers with long flow times (ca. 15 mins.) and large dilution bulbs - see Section II). The possibility of the curves having been caused by capilliary adsorption, was eliminated by checking selected results with a viscometer constructed with four separate capilliaries of differing diameters.

The viscosity measurements are confirmed from the ultra-centrifugal sedimentation coefficients (figure 11). In agreement with the viscosity data the sedimentation-coefficient-concentration relationship is relatively linear for 10^{-1}, 10^{-2}, and 10^{-3} M saline. For 10^{-4} and 10^{-5} M saline and water the polyelectrolyte effect is no longer swamped by surrounding electrolyte and a much more complicated relationship arises.

The above results indicate that, although phosphate groups are present on only approximately one, of every three hundred glucosyl residues in amylopectin, the effect caused by their ionisation upon the amylopectin configuration is substantial. Amylopectin behaves as an uncharged molecule in swamping amounts of saline as shown by viscosity and sedimentation. These results explain the large changes in the behaviour of starch pastes in the presence of electrolytes, reported by Nutting (1951).

Aggregation of Amylopectin: Since most of the reports of the very large molecular weights of amylopectin are based upon light-scattering measurements, it is possible that these could be caused by relatively small amounts of high molecular-weight aggregates. These would affect the weight-average molecular weight of such a broad distribution to a very disproportional extent. Accordingly,

a /

a sample of amylopectin was measured, by light-scattering in water, and three hydrogen bond-breaking solvents - 1% sodium lauryl sulphate solution, 8 M Urea and 15% magnesium chloride solution. It was found (figure 12) that, allowing for the differing dn/dc values, there was no change in the molecular weight, thereby eliminating the possibility of hydrogen-bonded aggregates.

In order to check that all the molecular species were similar over the very broad molecular-weight range present in amylopectin and that there was no change in the degree of branching with increase in molecular size, some amylopectins were degraded with β-amylase (see p. 83) and the weight-average molecular weight ($\bar{M}w$) of the β-amylolysis limit dextrin was compared with that of the original substance. If all the species are degraded to the same extent the percentage change in $\bar{M}w$ should equal the β-limit. This was, in fact, found (within experimental error).

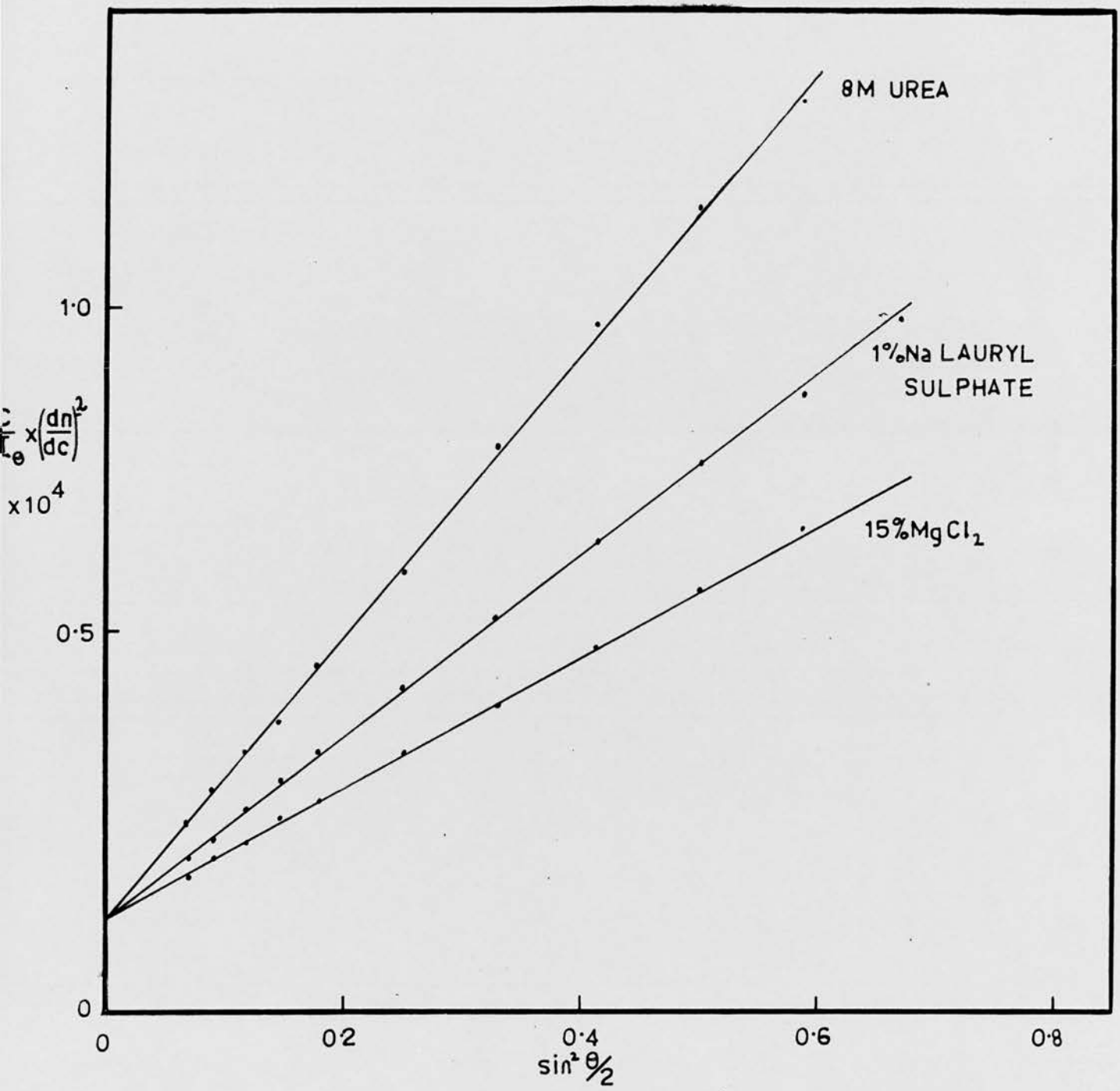
Amylopectin	% change in $\bar{M}w$	β-limit %
1	53	55
2	56	58
3	57	56

These results also suggest that if an aggregation is present then it involves all molecular species since if aggregation were limited, it would appear unlikely that the extent of any aggregation, persisting after a 45% loss of weight on β-amylolysis, would be equivalent to that before treatment with the enzyme.

The results of light-scattering measurements on amylopectin-acid which have been extended from the usual range of 5-20 x 10^5 g/ml.

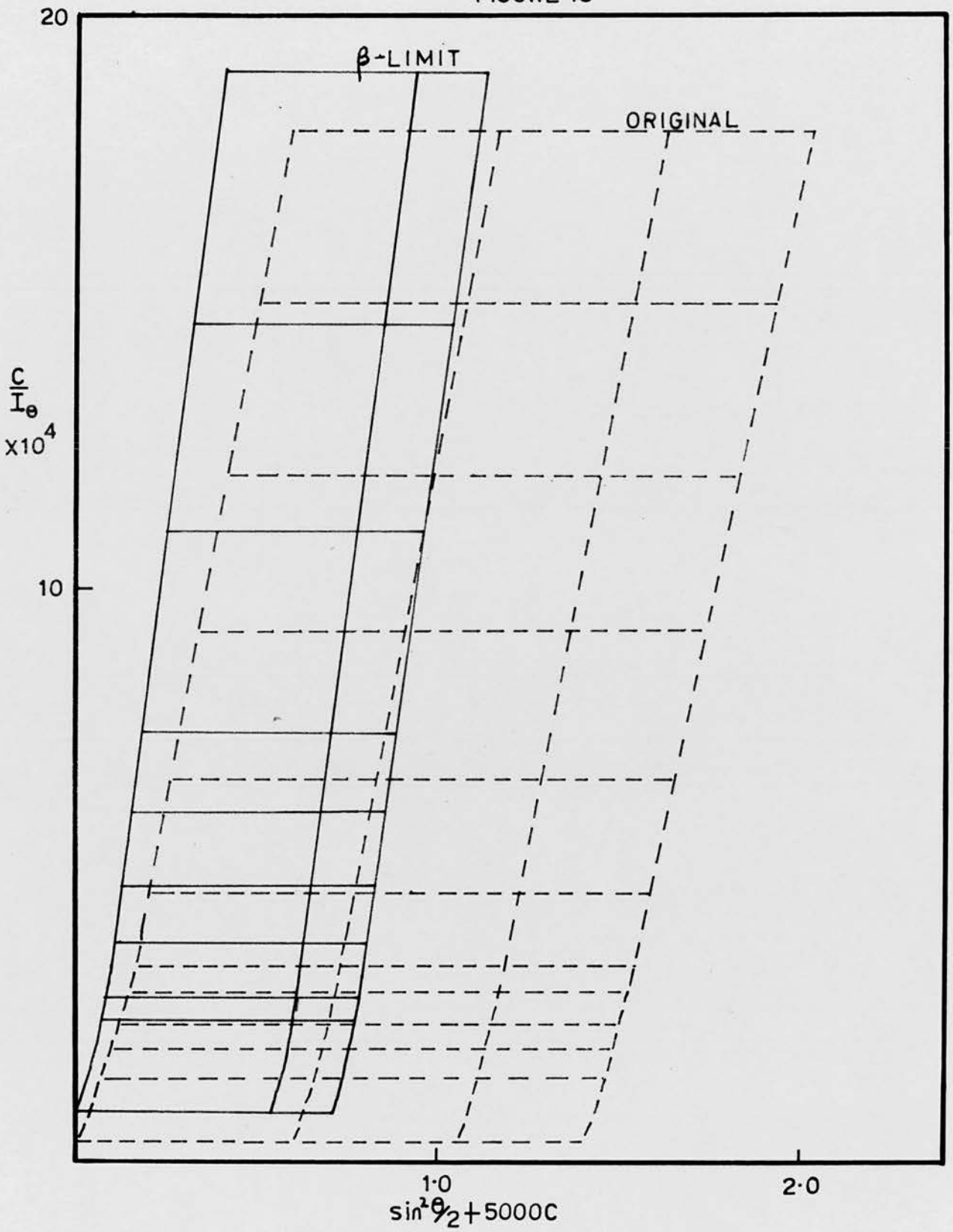
up /

FIGURE 12



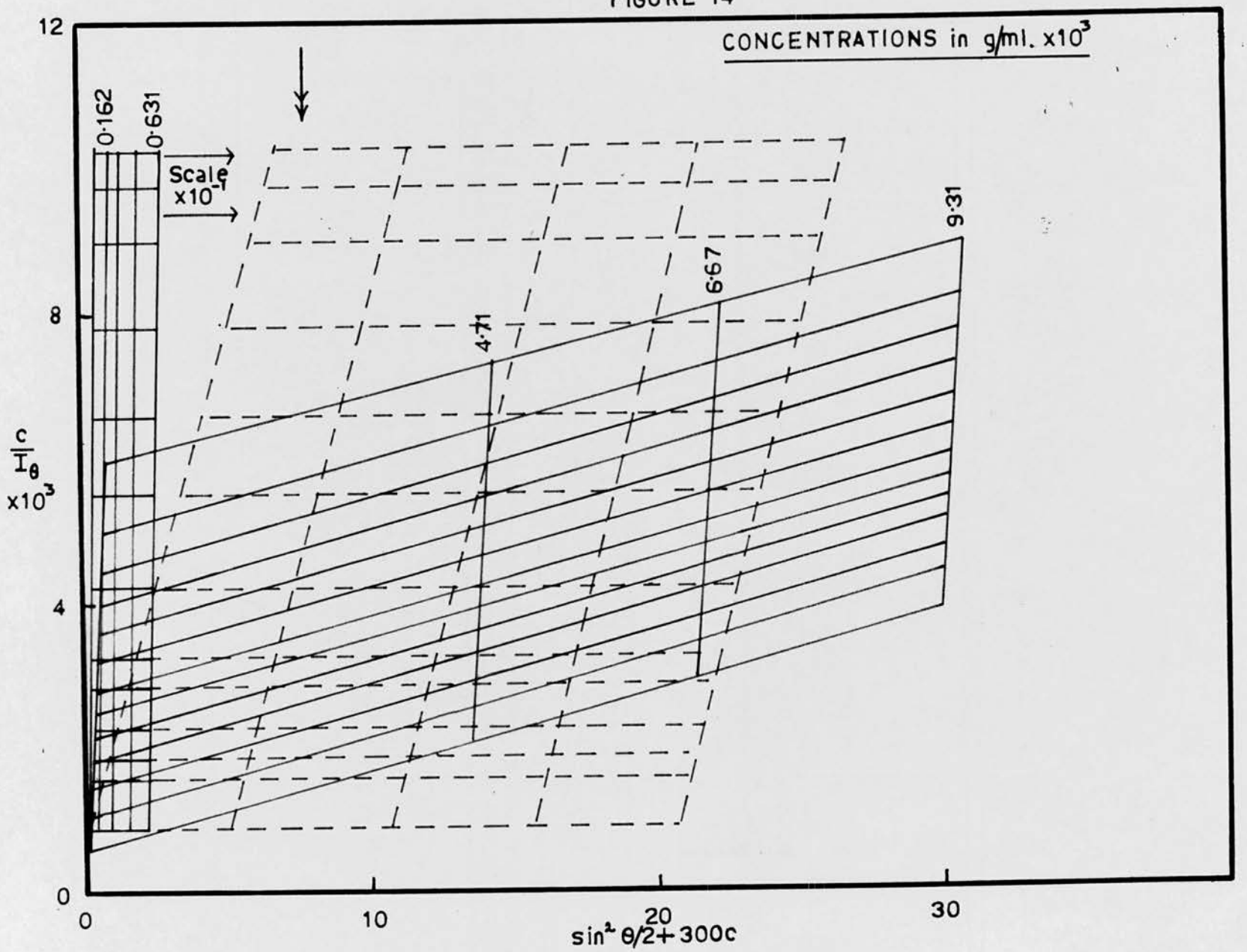
AMYLOPECTIN in DISAGGREGATING SOLVENTS — ZERO CONCENTRATION LINES of ZIMM PLOTS.

FIGURE 13



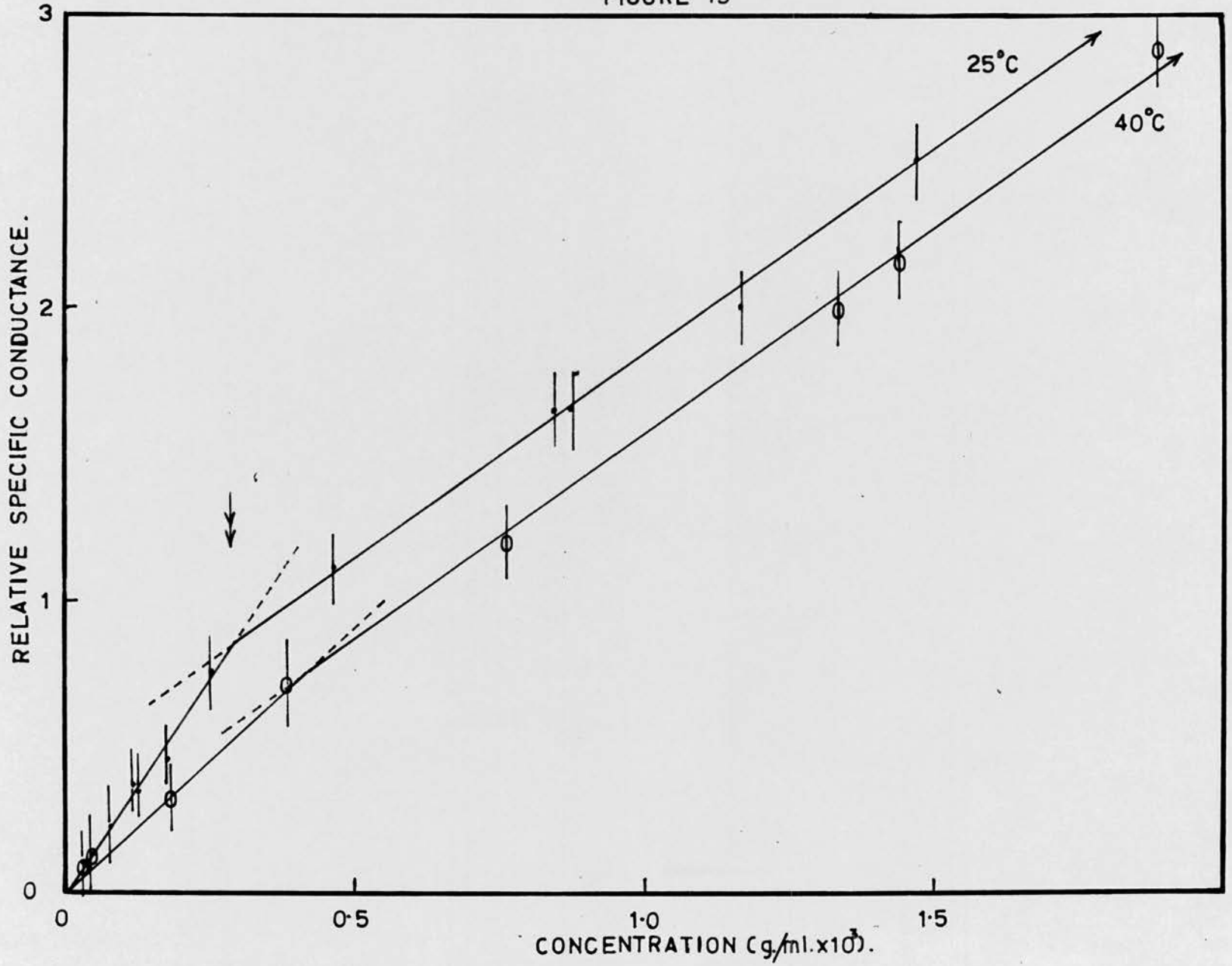
ZIMM PLOTS of AMYLOPECTIN and its β-LIMIT DEXTRIN.

FIGURE 14



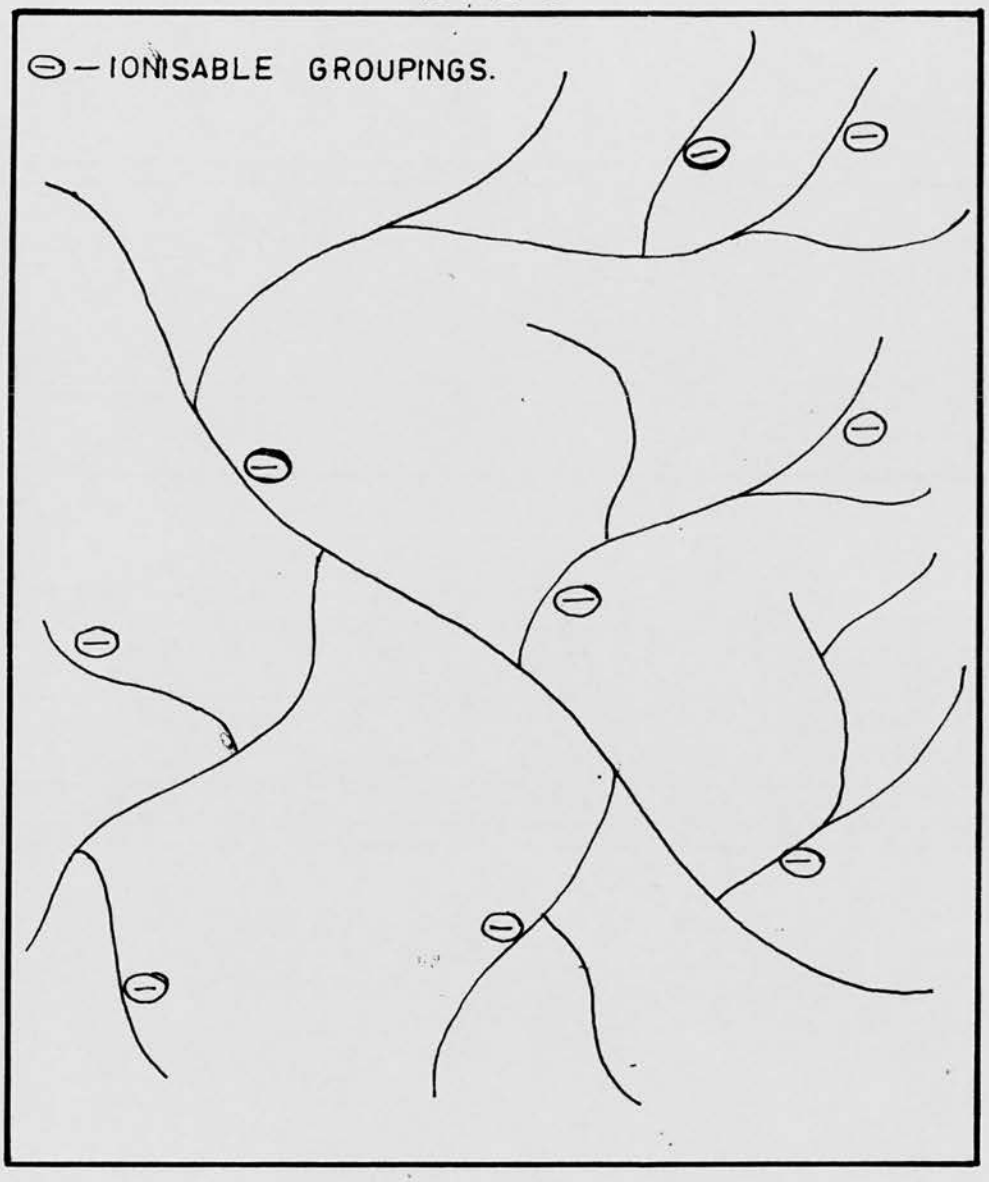
ZIMM PLOTS of AMYLOPECTIN ACID (LARGE CONCENTRATION RANGE).

FIGURE 15



CONDUCTIVITY of AMYLOPECTIN ACID.

FIGURE 16



MODEL for AMYLOPECTIN ACID.

up to 9.3×10^{-3} g/ml. are shown in figure 14. The data are best represented by two Zimm-Plots; one in the usual concentration range has no virial coefficient and the second at high concentrations has a distinct virial coefficient and a substantially lower intercept on the C/I_θ axis.

A corresponding change in the properties of amylopectin-acid is noted in the conductivity data. This is shown in figure 15. The concentration region where the effect occurs is markedly altered by increasing the temperature. (Experimental procedure as in Section II).

DISCUSSION.

From the above experimental data it can be seen that amylopectin-acid apparently behaves differently in two separate concentration regions. At "low" concentrations (i.e. up to 2×10^{-3} g/ml.) it behaves as a polyelectrolyte, while at higher concentrations the anomalous light-scattering and conductivity data suggest the formation of colloidal aggregates.

The polyelectrolyte behaviour of amylopectin at "low" concentrations has been well-characterised by the light-scattering, viscosity, and sedimentation data. These results suggest the model for amylopectin illustrated in figure 16. Repulsion of the negative charges on the orthophosphate groups, which will depend upon the strength of the surrounding ionic media, will cause expansion of the molecule. A quantitative approach to this work will only be achieved when some more accurate method of estimating the percentage phosphorus content can be found. At present the accuracy /

accuracy of the estimation is no better than 10%.

However the present work has shown that

(i) the electric charge first noted in starch by Samec (1935) is due to the presence of orthophosphate groups in amylopectin.

(ii) the orthophosphate groups in amylopectin extracted from the tuber can be converted to their free acid form by a mild ion-exchange technique which involves no danger of molecular degradation.

(iii) the results of titration of amylopectin-acid are essentially the same as those obtained for free orthophosphoric acid (and for electrodialysed starches (Briggs and Hanig (1946))), thereby confirming the nature of the phosphorus in amylopectin.

(iv) pronounced polyelectrolyte behaviour is caused in the amylopectin by the repulsion of the ionised phosphate groups.

In the "high" concentration regions the conductivity and light-scattering results suggest amylopectin forms an aggregate. The break in the relative specific conductivity versus concentration line indicates that at a specific concentration (2.9×10^{-3} g/ml., at 20°C, phosphorus content 0.06%) the number of free charged species decreases abruptly. This may be due to the formation of an aggregate e.g. a dimer, when the number of species available for conductance would be halved. Parallel behaviour has been found for other polyelectrolytes, notably detergents (McBain (1950)).

The formation of such an aggregate in amylopectin-acid would explain the light-scattering Zimm-Plot obtained at high concentrations (figure 14). The concentration region where the conductivity break /

break occurs is indicated on the graph and it can be seen that the results on either side of this break are best treated by the drawing of two separate Zimm-Plots. The extrapolation of the two Zimm-Plots separately shows that in the high concentration region the amylopectin has a distinctly higher molecular weight (92×10^6) than in the lower one (73×10^6). The fact that the molecular weights are not simple multiples suggests that all the molecular species do not aggregate; perhaps the higher molecular weight species are already too large.

Further evidence for aggregation is provided by the conductivity measurements at a higher temperature ($40^{\circ}C$, figure 15) which shows a movement of the "break" in the relative specific conductivity-concentration line to higher concentrations. This behaviour is again paralleled by that of detergent aggregates.

In conclusion, it is suggested that the solution behaviour of potato amylopectin is much more complex than was previously thought. In particular, there appears to be a distinct tendency amongst amylopectin molecules to form aggregates at concentrations above ca. 3×10^{-3} g/ml. Future experimentation should be directed towards determining the effect of increasing concentration upon a colligative property such as the osmotic pressure.

Experimental Studies of Starch Biosynthesis.

Experimental studies of the complex biosynthesis which must exist in the starch granule to produce two such structurally distinct components as amylose and amylopectin can be divided into three separate types:

(a) the enzyme systems involved

(b) the fine structure of the starch granule

(c) the physico-chemical characteristics of the products of biosynthesis.

The first two types of study have been far more extensively investigated than (c). Typical of (a) is the work of Whelan *et al* on D-enzyme and phosphorylase and the work of Leloir and his collaborators on amylose synthetase. This work is referred to extensively in the next Section as are the studies on the physical nature of the starch granule itself, notably by Badenhuizen. Studies of the third type have mainly been confined to the physical and chemical properties of starch isolated at successive times during the growth of the plant. Apart from the work of Greenwood and Thomson (1962) on starch isolated from peas, there is little evidence in the literature as to whether changes occur in the fine-structure of the components during growth. Since the products of such a complex biosynthesis have as much importance as the enzymes involved in the formulation of any theory which could adequately describe the process involved, a study of the changes in the amylose and amylopectin of the growing potato tuber was initiated.

Also from the point of view of biosynthesis, it is well-known that some varieties of the potato *Solanum Tuberosum*, tend to produce /

produce fruit or berries, which in common with many other fruit contain starch. Any differences in the characteristics of starch isolated from the berry and the tuber would be of great potential interest to any theory of biosynthesis.

AMYLOPECTINS FROM POTATO BERRIES. This starch had not been investigated until the work of Greenwood and MacKenzie (1963). These workers isolated starch from three varieties of potato, Solanum Tuberosum, in sufficient quantities for detailed investigations.

The properties of the berry starches were compared with those of starch from the corresponding tuber. Differences in nitrogen-content, phosphorus content, gelatinisation temperature and iodine affinity of the two types of starches were not profound. It was also found that the berry and tuber starches were degraded to similar extents by acid but on attack with amylolytic enzymes, the berry starches were found to be eroded more easily.

On fractionation of the granules into their amylose and amylopectin components, it was found that all the amylose samples had β-amylolysis limits of less than 100%. The molecular size, as indicated by viscosity measurements, of the berry-amyloses were slightly smaller than the tuber-amyloses. The β-amylolysis limits, and average-lengths of unit chain of all the amylopectin samples were similar. It was therefore of some interest to measure the molecular weights of these amylopectins.

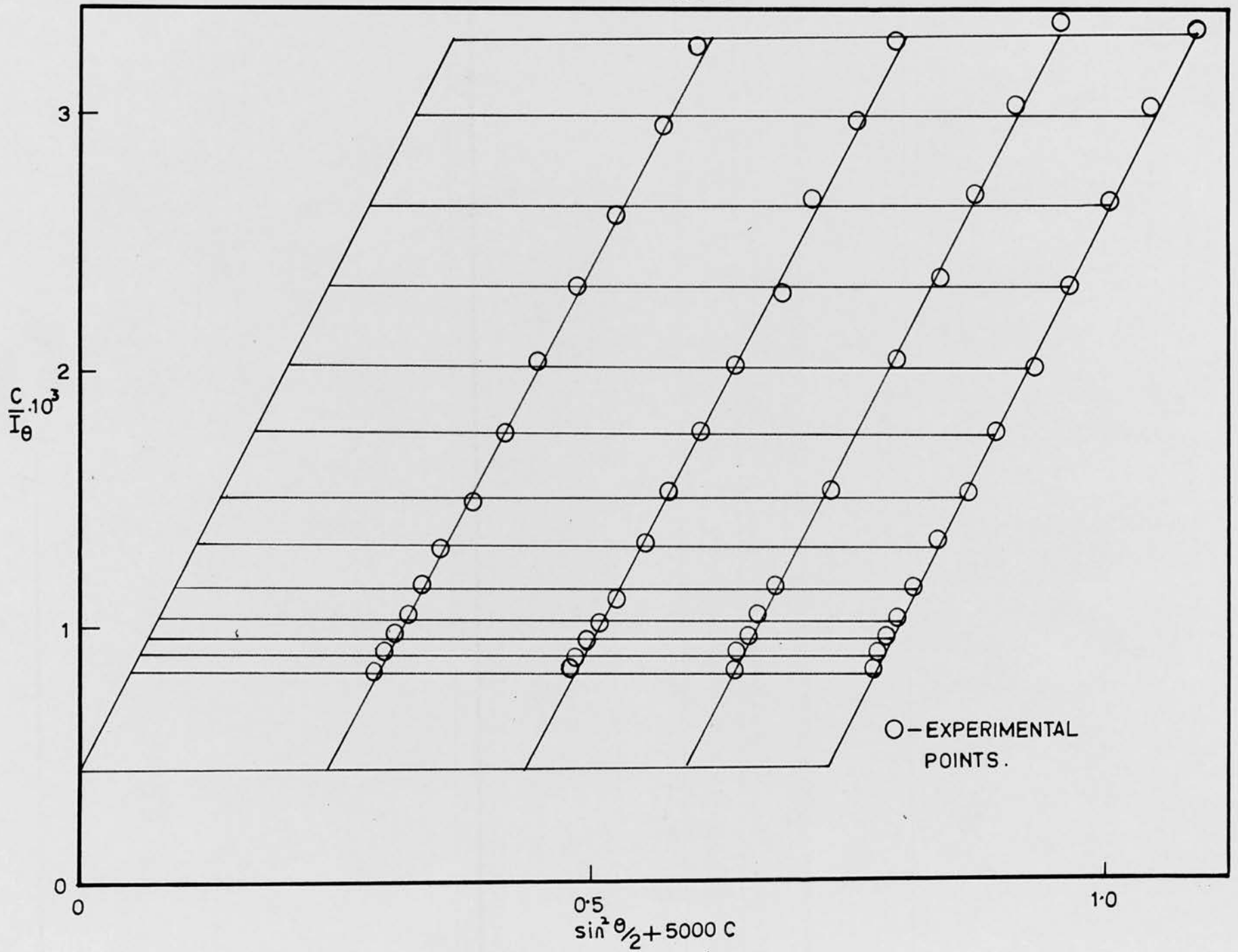
Experimental.

The molecular weights were obtained from light-scattering of the amylopectins in 0.1M sodium chloride, by the method indicated previously.

Results.

A typical Zimm plot is shown (Figure 17). Results were as shown /

FIGURE 17



ZIMM PLOT for Tuber AMYLOPECTIN (var. Dr. MacIntosh) in 0·1M NaCl.

shown below:

Variety	Source	Molecular Weight X 10^{-6}	Radius of Gyration (Å)
Dr. MacIntosh	Tuber	110	2280
Dr. MacIntosh	Berry	55	1810
Pentland Dell	Tuber	8	1210
Pentland Dell	Berry	35	1650

The molecular weights and radii of gyration are of the typical size of amylopectins from potato sources. The variation is probably due to differences in maturity. It appears, then, that berry and tuber starch are very similar. No significant difference is apparent from a biosynthetic viewpoint and a radical change in the method of biosynthesis of starch in the berry compared to that in the tuber, is not very likely.

PROPERTIES OF THE COMPONENTS OF STARCHES FROM THE GROWING POTATO TUBER. (in collaboration with S.L. MacKenzie).

Previous studies have established that during growth there are changes in the amount of starch, and the characteristics of the starch granules, stored in the plant. The species investigated were maize (Wolf et al (1948)) Erlander (1960)), sweet corn (Maywald et al (1955)), barley (Harris et al (1958)), peas (Greenwood et al (1962), and tobacco-leaves (Matheson et al (1962), (1963)). These studies indicate that as the plant matures, its starch-content increases with a concurrent increase in both the average size of the granule and the amount of the amylose component that it contains. This growth pattern conflicts with that indicated by Halsall et al (1948) for the case of potato starch where the percentage amylose content remained constant for two varieties of growing tuber. It is also in disagreement with the results of Thomson (1961).

It was therefore of interest to study the physical and chemical properties of starch isolated from the growing potato tuber and the changes in the fine structure of the amylose and amylopectin components.

EXPERIMENTAL

Growth and Isolation of the Starches.

The potatoes (variety Pentland Crown) were grown at the Scottish Plant Breeding Station, Pentlandfield, Roslin, Midlothian in the 1962 - season. The tubers were harvested at intervals of ten days after the appearance of leaves on the plants, and were separated into sizes varying from less than 1 cm. to 15 cm. in longitudinal diameter. Starch was isolated from the different sized tubers as described in Section I, and was purified by the method of Banks and Greenwood (1959).

Characterisation of the Starches.

The granular size distribution, the number-average particle diameter, the percentage of phosphorus, the average gelatinisation temperature, and the determination of the iodine affinity of the starches were determined by MacKenzie as described by Banks and Greenwood (1959).

Fractionation of the Starches and Characterisation of the Amylose and Amylopectin Components. (by S.L. MacKenzie).

The starch samples were fractionated conventionally (Banks et al (1959b)) after liquid ammonia pretreatment.

Iodine affinities, percentage phosphorus contents, viscosities (in 1M KOH, 25°C), β-amylolysis limits and periodate oxidations were measured as detailed by MacKenzie (1964).

Light-scattering Measurements on the Amylopectins.

Light-scattering measurements were obtained as detailed in Section II. The samples, in 0.1M sodium chloride, were measured in /

in cylindrical cells over the angular range 30°- 120°. All measurements were corrected for solvent-scatter.

Results.

All results showing trends which are large enough to be biosynthetically meaningful are illustrated in Table 3 . Other properties either varied randomly or any trend was of the same order as the experimental error involved. Typical of the former were the phosphorus (ca. 0.05%) and nitrogen (ca. 0.01%) contents of the starch granules, and typical of the latter was the chain-length of the amylopectins as determined from periodate oxidations which varied from 26 (0-1cm. tuber) to 22 (15-16cm. tuber). Full details of all these results are described elsewhere. (MacKenzie (1964)).

The results showed that - in agreement with the results of the previous biosynthetic studies (see Thomson (1961)) - as growth proceeds the starch-content of the granule increases and there is a simultaneous increase in the percentage amylose content of the granules (as shown by iodine titration measurements). The fine-properties of the separated starch components have never been so intensively measured previously. As the growth proceeds the molecular size of the amylose increases, Table (b), as shown by viscosity measurements. The approximate degree of polymerisations calculated from these measurements are based on the relation of Cowie and Greenwood (1957). The β-amylolysis limits of the amyloses indicate that, as the starch granules mature, the amylose component becomes /

TABLE 3

(a) PROPERTIES OF THE STARCH GRANULES

Tuber size (cm.)	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	14-15	15-16
Starch content(%)	5.0	5.6	6.4	8.3	9.2	11.0	13.4	17.0	17.5	17.5	18.0	-	-
Amylose*(%)	12.5	13.2	13.9	14.6	16.4	17.2	18.2	18.5	19.0	19.2	19.8	20.0	20.0

(b) PROPERTIES OF THE AMYLOSE COMPONENT

β-limit	92	90	90	88	88	87	85	84	84	83	83	81	81
$\overline{D.P}$ ∠	2200	1100	1400	1600	2100	2800	3100	3200	3300	3600	3700	4000	3900

(c) PROPERTIES OF THE AMYLOPECTIN COMPONENT

Molecular Weight* ($\times 10^{-6}$)	9	-	-	23	-	-	35	-	-	37	-	60	130
$\overline{(A)^2}^{*//}$	1040	-	-	1290	-	-	1510	-	-	1450	-	1600	2120

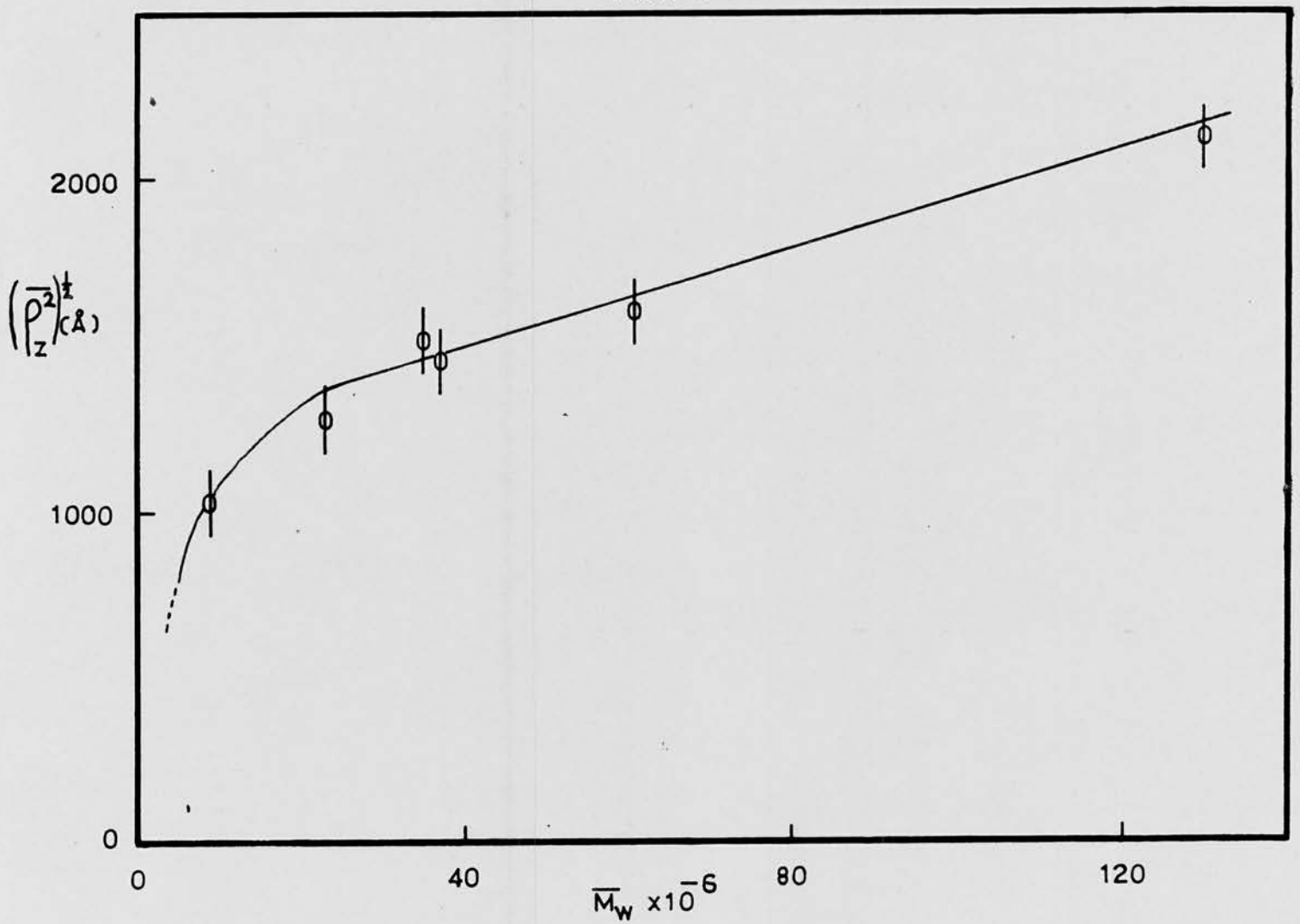
* From iodine affinity measurements

∠ From viscosity measurements

* From light-scattering measurements

// Root-mean-square end-to-end distance

FIGURE 18



VARIATION of root-mean-square RADIUS of GYRATION with $\overline{M}_w$ for AMYLOPECTINS ISOLATED at VARIOUS STAGES of GROWTH.

becomes less susceptible to degradation by the enzyme i.e. some "barrier" is being introduced. This trend in β-amylolysis limits affords a strong indication that the barrier is a natural one and not an artificial product of the fractionation procedure. (See also Section VI).

The light-scattering results on the amylopectin components, Table 3 (c), indicate that there is a profound increase in the molecular weight from 9×10^6 to 130×10^6 with increase in maturity. Data were evaluated by the method of Zimm (1948) and in agreement with earlier work in these laboratories (Greenwood & Thomson (1962)), the second virial coefficient in 0.1M sodium chloride, in the concentration range studied ($5 - 15 \times 10^{-5}$ g/ml.), was negligible. With the increase in molecular weight there was a corresponding increase in the root-mean-square end-to-end distance. On plotting the latter values against the molecular weight an approximately linear relationship is obtained. (Figure 18). This may indicate that the degree of branching of the amylopectin molecules remains approximately constant as the molecular size increases.

Discussion.

The above results show that with increase in maturity of potato starch there is - in addition to changes in the size and properties of the granule - a profound alteration in the fine structure of the components, especially amylopectin. The biosynthesis of amylose and amylopectin is, therefore, not a simple process. Current theories of starch biosynthesis are those of Whelan /

Whelan (1958, 1963) and Erlander (1958). These results suggest that biosynthesis is more complicated than either of these theories indicate. This is discussed in the following Section.

SECTION IV.

THEORIES OF STARCH BIOSYNTHESIS.

SECTION IV

Theories of Starch Biosynthesis.

Introduction.

It was found that the results obtained in collaboration with Dr. S.L. Mackenzie (Section III) were not consistent with any of the current biosynthetic theories. Consequently a critical evaluation of current biosynthetic knowledge was undertaken and a new theory, with which our results agree rationally, has been proposed.

The biosynthesis of starch within the granule is a complex process utilising both a variety of enzymes and of substrates. Recent advances in enzymic studies - notably by Leloir and his collaborators (1957, 1959, 1960, 1961,a,b., 1962) - have shown the presence of a biosynthetic nucleotide pathway in the starch granule in addition to the well-established phosphorylase mechanism. Whelan (1963) has incorporated these two pathways into a biosynthetic scheme which, however, does not readily allow for the increase in amylose content with granule size (or maturity) observed by some authors (Section III, Thomson (1961), Bice et al (1945), Harris et al (1958), Erlander (1960)), and which ignores the physical nature of the starch granule itself.

The facts which any biosynthetic theory must reconcile are described in the first three subsections.

A. /

A. Physical Nature of the Starch Granule.

Microscopically the starch granule is a transparent layered object, which under crossed Nicols exhibits birefringence. This birefringence is assumed to be due to parallel arrangements of the outer branches of the amylopectin molecules since it is also noted for "waxy" maize granules (ca. 100% amylopectin). These layers were originally thought to have arisen from diurnal variation in biosynthesis (Meyer, (1895)), but this has since been proved conclusively not to be the case. (Roberts et al (1954), Mes et al (1954), Bünning et al (1954)). It has been firmly established that the layers are formed by apposition i.e. they are laid down successively from the outside. (Badenhuizen et al (1956), Yosida et al (1958a, 1958b). Even finer layering has been shown from the electron - microscopy studies of Buttrose (1962).

Most starch granules are found in those organs of the plant which store starch for a long period e.g. tubers, bulbs, seeds etc. Problems concerned with starch formation in amyloplasts are of a genetical nature. The shape and average size of the starch granules, the shape of their molecules, the ratio of linear to branched components, and the degree of molecular association will all be influenced by separate genes.

B. Enzymes present in the Starch Granule.

The enzymes which have been found in plant sources are shown in Table 4. Their mode of action of the more important ones will be outlined.

PHOSPHORYLASE /

TABLE 4

ENZYMES INVOLVED IN STARCH BIOSYNTHESIS

ENZYME	FUNCTION	REFERENCE
Phosphorylase	Synthesis and Degradation of 1→4 links	Hanes (1940)
Amylose Synthetase	Synthesis of 1→4 links	Leloir et al (1957)
D-ENZYME	Synthesis and Degradation of 1→4 links	Peat et al (1956)
Q-ENZYME	Synthesis of 1→6 links	Haworth et al (1944)
T-ENZYME	Synthesis of 1→6 links	Abdullah et al (1960)
R-ENZYME	Degradation of 1→6 links	Hobson et al (1951)
α-Amylase	Degradation of 1→4 links	
β-Amylase	Degradation of 1→4 links	

PHOSPHORYLASE (P-enzyme) has been well characterised since its isolation in 1940 from potato juice by Hanes. P-enzyme synthesises 1 → 4 linked α-D-glucopyranosyl chains using glucose-1-phosphate as the donor and linear chains of glucose units as the acceptor molecules. To be effective, the acceptor (or primer) molecules must contain at least 4 glucose units. (Whelan et al (1954), Bailey et al (1961)). Synthesis by P-enzyme has been shown to follow a multichain action pattern (Whelan et al (1954)). The enzyme also functions in a degradative manner breaking 1→4 links in a glucose chain from the non-reducing end-group in a stepwise manner. The equilibrium involved has been investigated (Hanes, (1940)).

$$\text{Glucose-1-P} + (\text{Glucose})_n \rightleftharpoons (\text{Glucose})_{n+1} + \text{Inorganic Phosphate}$$

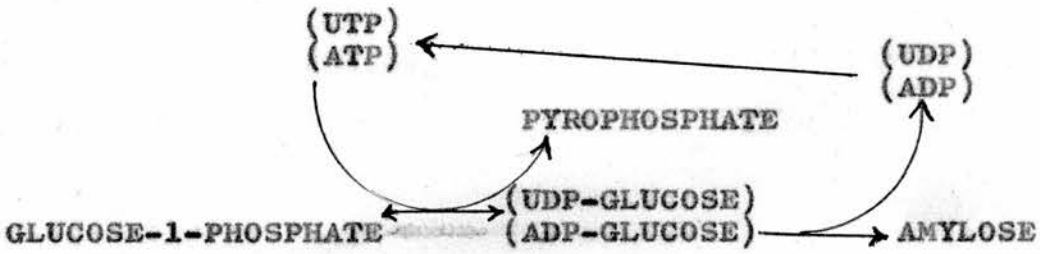
The equilibrium ratio of Glucose-1-phosphate to inorganic phosphate indicates a strong preference for the enzyme to function in a degradative rather than a synthetic manner i.e.

pH	5	6	7
$\frac{\text{Glucose-1-P}}{\text{Inorg. P}}$	10.8	6.7	3.1

This point however appears to have been overlooked, and until the discoveries of Leloir et al it was thought that phosphorylase was the main enzyme involved in the formation of amylose. (See e.g. Whelan 1956-7).

AMYLOSE SYNTHETASE. An alternative pathway of amylose biosynthesis was reported by Leloir and Cardini (1957) and is catalysed by Uridine diphosphate glucose (UDPG) starch transglucosylase (amylose /

(amylose synthetase) and is apparently non-reversible. The acceptor requirements for this enzyme are the same as for phosphorylase i.e. a primer of D.P. ≥ 4 whilst the donor molecule is UDPG. Subsequently Recondo and Leloir (1961) found that adenosine 5-(D-glucosyl pyrophosphate) (ADP) will also act as a donor for this enzyme. The rate of transfer of glucose-1-phosphate to starch by this mechanism is approximately ten times more rapid than by the UDP mechanism. The two pathways are essentially similar



In this work it was found to be difficult to separate the enzymic protein from the starch granules and, in fact, a suspension of the granules does function efficiently as an enzyme preparation. However, some degree of separation has been achieved by Pottinger and Oliver (1962) by isolating the granules in 0.5M sucrose-citrate medium rather than water. Nevertheless it does appear that the enzymic protein is found on the granule surface rather than in the interior.

The glucose transferred to starch by the amylose synthetase enzyme, utilising either UDPG or ADPG, was found by radiochemical measurements to be distributed between the amylose and amylopectin components (Recondo and Leloir (1961)). The transfer is irreversible.

D-ENZYME./

D-ENZYME. In the three syntheses mentioned above, an acceptor molecule of at least four glucose units is required. It is thought that these may arise by the action of the disproportionating enzyme, D, which functions as follows (Peat et al 1956a, 1956b),

2 maltotriose $\xleftarrow{\text{D-Enzyme}}$ Glucose + maltopentaose

D-enzyme, a transglucosylase, is present in appreciable amount in potato juice. It has no action on glucose or maltose but for higher maltodextrins functions as shown above. Some of the oligosaccharides synthesised from the maltodextrins are long enough to stain with iodine.

Q- and T-enzymes (Haworth et al (1944), Abdullah et al (1960)).

The enzymes so far mentioned, function in a purely linear manner i.e. they synthesise amylose-like α-1 → 4 linked molecules. Other enzymes must be present to synthesise the α-1→6 links, which give amylopectin its typical branched structure. The function is performed by two transglucosylase enzymes, Q and T. Q-enzyme splits an α-1 → 4 link in a donor molecule of amylose and joins the split fragment to an acceptor molecule of amylose through an α-1 → 6 bond. Q-enzyme action on amylose is only rapid when the amylose has a D.P. greater than 40 glucose units. T-enzyme performs exactly the same reaction as Q-enzyme but it acts readily on small oligosaccharides which Q-enzyme will not. A typical reaction is,

maltose + maltose $\xrightarrow{\text{T-enzyme}}$ Panose + glucose

The reaction is irreversible.

C. /

C. Current Biosynthetic Theories.

It would appear that the above-mentioned enzymes can explain in a relatively simple manner the biosynthesis of amylose and amylopectin. Amylose is formed by the concurrent action of phosphorylase, D-enzyme and UDP- and ADP- glucose starch transglucosylase. Amylopectin is formed by the action of Q- and T- enzymes on the linear substrates provided by the above enzymic mechanisms. This synthesises 1→6 links which then continue to grow by the 1→4 link synthesising enzymes, interspersed by occasional branching reactions. (The frequency of which are probably determined by the steric requirements of the enzyme).

These separate syntheses are well-known and have been shown to occur *in vitro*. e.g. Husemann and Pfannemuller's (1954) preparation of synthetic amylose.

Unfortunately the concurrent syntheses of amylose and amylopectin in the starch granule cannot be explained in this manner, since mixtures of linear and branching enzymes inevitably give branched material, albeit with various degrees of branching dependant on the branching to linear enzyme ratio. This also has been shown from *in vitro* experiments (Barker *et al* (1950)).

Any theory of biosynthesis must reconcile therefore, these enzymic facts with the properties of starch isolated at various stages of growth and detailed in the previous section. (See also J. Thomson (1961), Bice *et al* (1945), Harris *et al* (1958), Erlander (1960)) i.e. increases in the starch content, the size of /

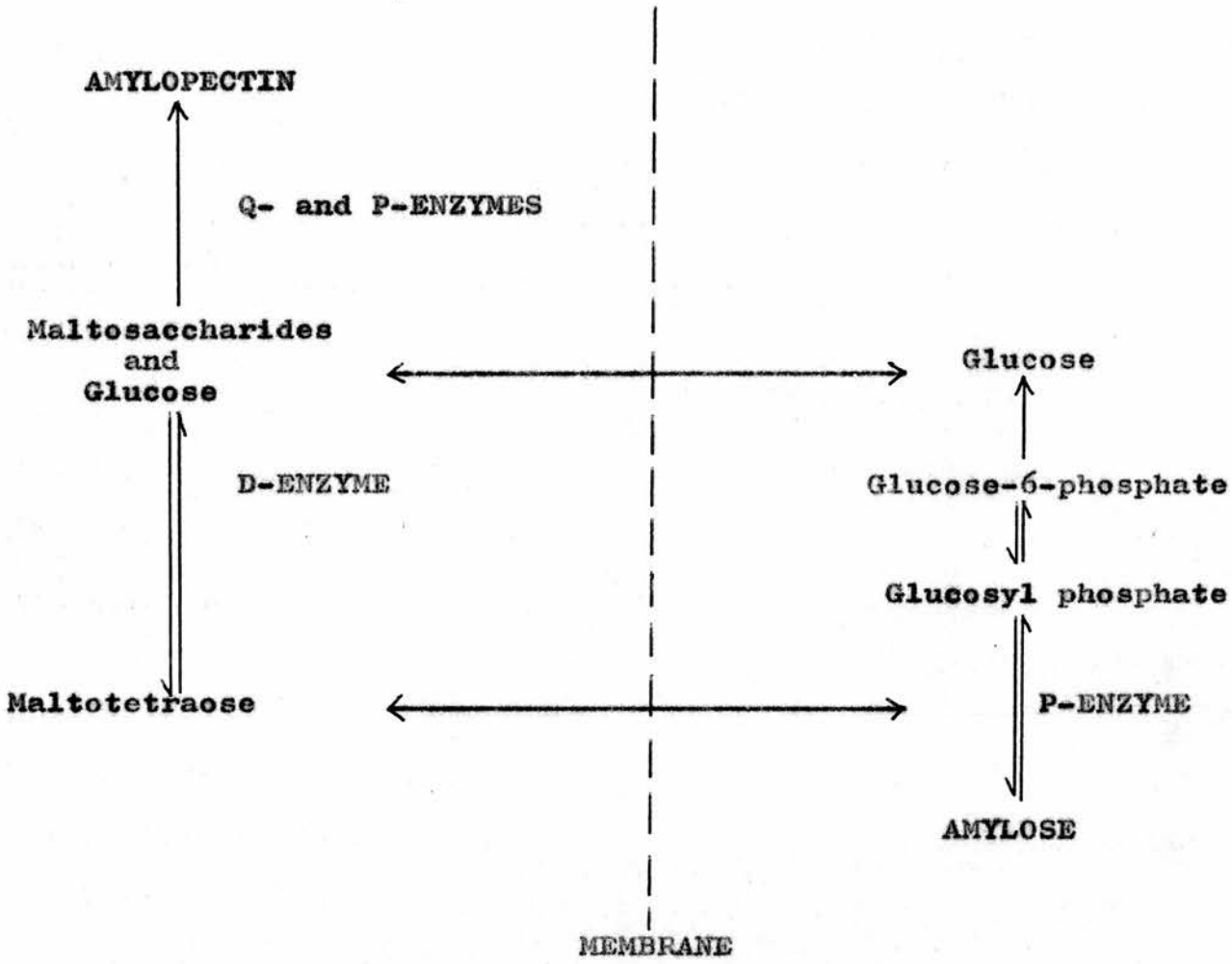


FIGURE 19 STARCH BIOSYNTHESIS THEORY (WHELAN (1958)).

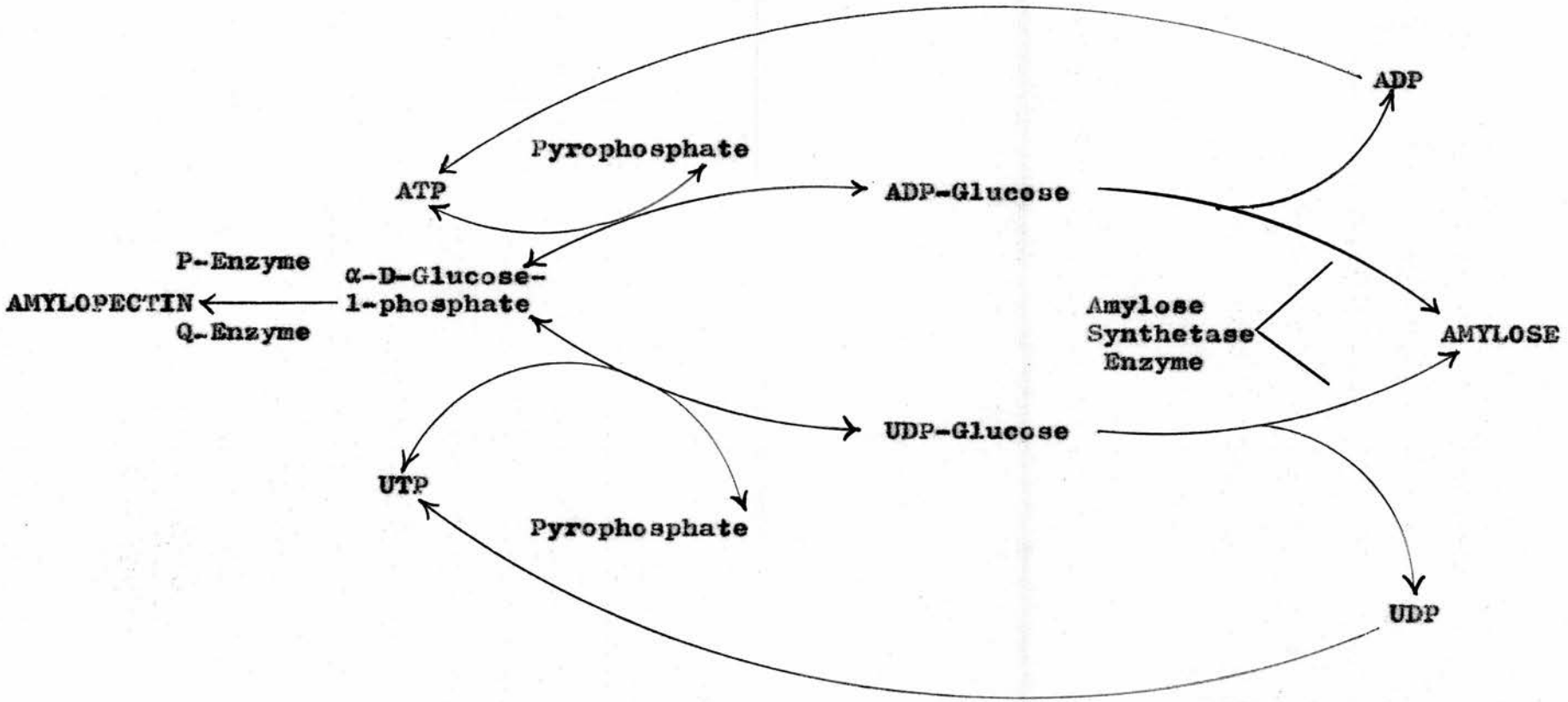


FIGURE 20 STARCH BIOSYNTHESIS THEORY (WHELAN (1963)).

of the granule, the % amylose content, the D.P. of the amylose, the chain length of the amylopectin, and the molecular weight of the amylopectin.

Current theories of starch biosynthesis are due to Whelan ((1958), (1963)) and Erlander (1958). The earlier Whelan theory postulates the presence of a membrane permeable to D-glucose and maltosaccharides $\leqslant 4$ only. On one side of the membrane are the "linear" enzymes while the other side has both "linear" and "branching" enzymes. The situation is illustrated in figure 19.

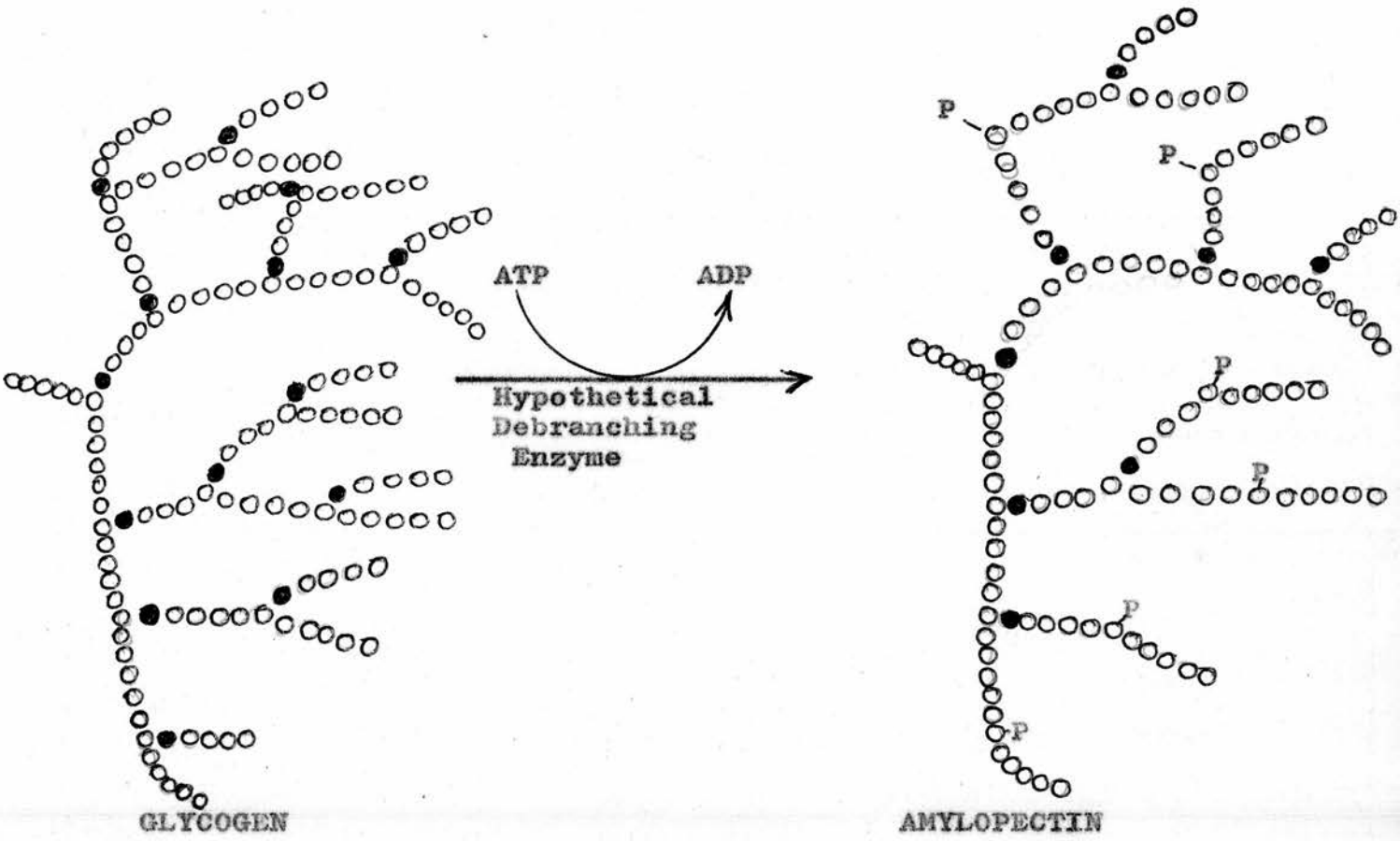
The 1963 - Whelan theory takes account of the observations of Leloir mentioned previously (P.58). This theory assumes that the pathways to amylose and amylopectin are not so closely linked as is normally presumed. In favour of this suggestion is involved the observation that normal maize granules contain starch synthetase but that waxy maize granules do not. (Nelson et al (1962)). It is thereby inferred that amylopectin must be synthesised by a mechanism other than the starch synthetase pathway. This assumption leads to the mechanism illustrated in figure 20. Again Whelan has invoked a semi-permeable membrane to separate the pure amylose-synthetising enzymes from the mixture of linear and branching enzymes required to produce amylopectin. He suggests that the membrane could be amylopectin itself by suggesting that "if the enzyme that synthesised amylose becomes surrounded by amylopectin then Q-enzyme would not be able to penetrate to the amylose, whereas the sugar nucleotide substrate (i.e. UDPG or ADPG), a much smaller molecule, could pass through."

However /

However there are several disputable points in these arguments. Firstly Frydman (1963) has reported ADPG-starch transglucosylase activity in waxy corn which would provide an alternative, very similar pathway, to that which would have been provided by the UDPG-starch transglucosylase which Nelson et al (1962) showed to be absent. Indeed there are indications that ADPG is the natural substrate, rather than UDPG (Murata et al (1963), (1964)). This observation is further substantiated by the evidence presented by Fuwa (1960) that amylose (or amylose-like dextrins) is present in waxy maize five to seven days after pollination. This obviously suggests that amylose and amylopectin are not indeed synthesised separately but by closely linked routes. This is also suggested by the results of McConnell et al (1958) who followed the process of starch synthesis in the wheat kernel by C^{14} tracer techniques, and also by the results of Barker et al (1949) and Nussenbaum et al (1951).

Another weakness in the Whelan theory is the arbitrary assumption of a semi-permeable membrane with extremely specialised properties. This seems unrealistic and certainly totally unconfirmed experimentally.

An alternative approach to the problem is provided by the theory of Erlander (1958). It is based on the observation that in sweet corn, a glycogen-type polysaccharide is found co-existing with granular starch. (Hassid et al (1941)). Erlander therefore suggests that glycogen may act as an intermediate in the synthesis of starch. The conversion of glycogen into starch is explained by /



(The chains enzymically removed join together to form AMYLOSE)

o Glucose unit connected by 1→4 linkage

● Glucose unit connected by 1→6 linkage

P Phosphate group

FIGURE 21 STARCH BIOSYNTHESIS THEORY (ERLANDER (1958)).

by assuming an irreversible enzymic debranching of the glycogen followed by the connecting of these linear debranched chains to form amylose. (See Figure 21). The partially debranched glycogen is amylopectin. The production of longer unbranched chains in the amylopectin allows this partially debranched glycogen and amylose to crystallise out of solution in the form of starch granules. The variation in the rate of crystallisation would cause the production of the granular rings.

This theory requires the existence of a highly specialised debranching enzyme of a type which has not yet been found to exist in any source including sweet corn. The well-known debranching enzyme of higher plants, R, and isoamylase, the yeast-debranching enzyme cannot hydrolyse interchain linkages in the interior of a glycogen molecule where the average internal chain length is only three or four units. (Peat et al (1954), Gunja et al (1962), Kjolberg et al (196)). Indeed even the very active α-amylase does not appear to penetrate the core of the glycogen molecule. (See Section VIII). Many other criticisms may be raised against this theory e.g. the lack of proof showing the existence of glycogen-like molecules in any plant other than sweet corn, the inability of Q-enzyme to introduce branching as dense as that found in glycogen, the inefficiency of glycogen as an acceptor for the linear-synthesising enzymes etc. (See Manners (1962) P. 394-5). This theory also, then, is considered unlikely.

B. /

D. Proposed Theory of Starch Biosynthesis.

The proposed theory of starch biosynthesis is illustrated in Figure 22. It is perfectly general but is capable of modification, as will be shown later, for particular cases such as that of waxy maize. The pathway involving the utilisation of sucrose is based upon the work of Akazawa et al (1964) who observed that in the presence of UDPG-sucrose, and UDPG-starch transglucosylases, and UDP there was a definite transfer of glucose from sucrose-C^{14} to starch molecules. These workers have also shown that fructose, the other half of the sucrose molecule is eventually converted to glucose-1-phosphate through the initial step of the phosphorylation reaction.

Under normal circumstances, it seems likely that phosphorylase is changed to its degradative role since inorganic phosphate will be produced by the UDP and ADP mechanisms and this would cause displacement of the phosphorylytic equilibrium in favour of the degrading reaction. (See P.58).

In order that the pathway may function efficiently, some limitation must be placed on the activity of Q-enzyme since, if it were perfectly free in its mode of action, the production of purely linear material could not be expected. Whelan, in his scheme, limits the randomness of Q-enzyme attack by postulating the existence of a semipermeable membrane or by amylopectin acting as one. However it would seem more reasonable to suggest that Q enzyme is associated with the surface of the granule, an assumption which is not entirely without justification. Both the experiments of /

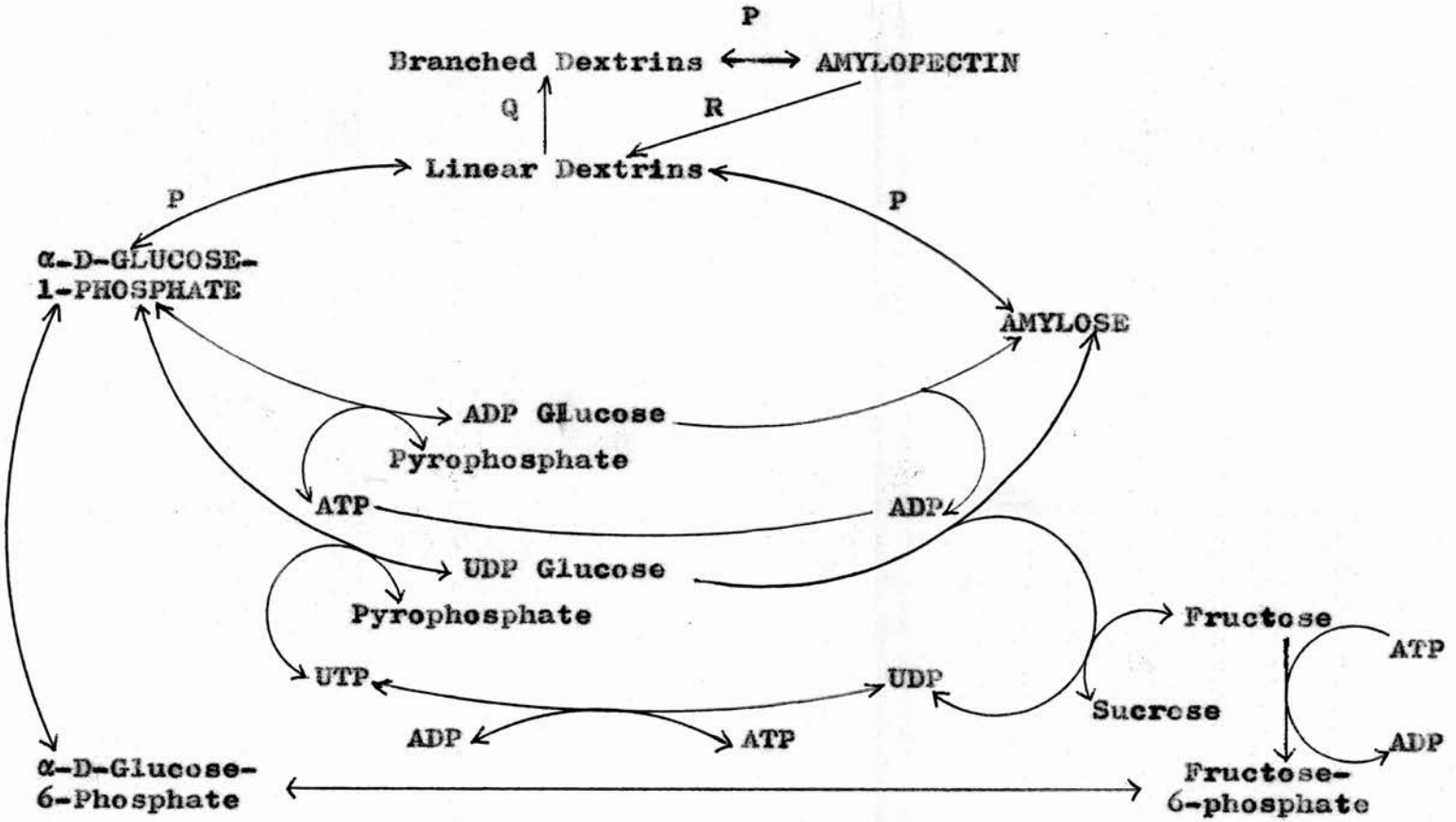


FIGURE 22 BIOSYNTHESIS OF AMYLOSE AND AMYLOPECTIN.

of Greenwood and Robertson (1954) and electron-microscopy (Hess et al (1955)) have indicated the presence of protein on the surface of the granule. It also appears from Leloir's experiments that the amylose synthetase enzyme is on the surface, because of the great difficulty involved in separating the enzyme from the granule. If this postulate is accepted (and it is certainly no less likely than Whelan's "semi-permeable membrane"), it is easy to visualise how amylose and amylopectin originate in the starch granule.

Figures 23 and 24 illustrate schematically the granule surface. "Linear" sites must, out of necessity, outnumber "branching" sites. "Linear" sites will synthesise exclusively $1 \rightarrow 6$ links by means of Q-enzyme. Only when "linear" and "branching" sites are in close proximity, will amylopectin molecules be produced, otherwise linear sites would produce amylose exclusively. Phosphorylase is presumed to be unbound, though if it were it would have no effect on the theory. These reactions will occur in the "annular" region and as they proceed this will expand as more polymer is produced. Finally a coacervate will form and the material will be precipitated forming a crystalline region. The reaction will then start again, by adsorbing enzymic protein from the amyloplast and proceed to form a new "annular" region. This process will continue until the amyloplast is exhausted of substrate or enzymic protein.

How does this theory agree with the experimental facts? Obviously the size of the granule and its starch content will increase as a function of the time of growth as has been found experimentally. (See, e.g., Section III). The increase in the percentage /

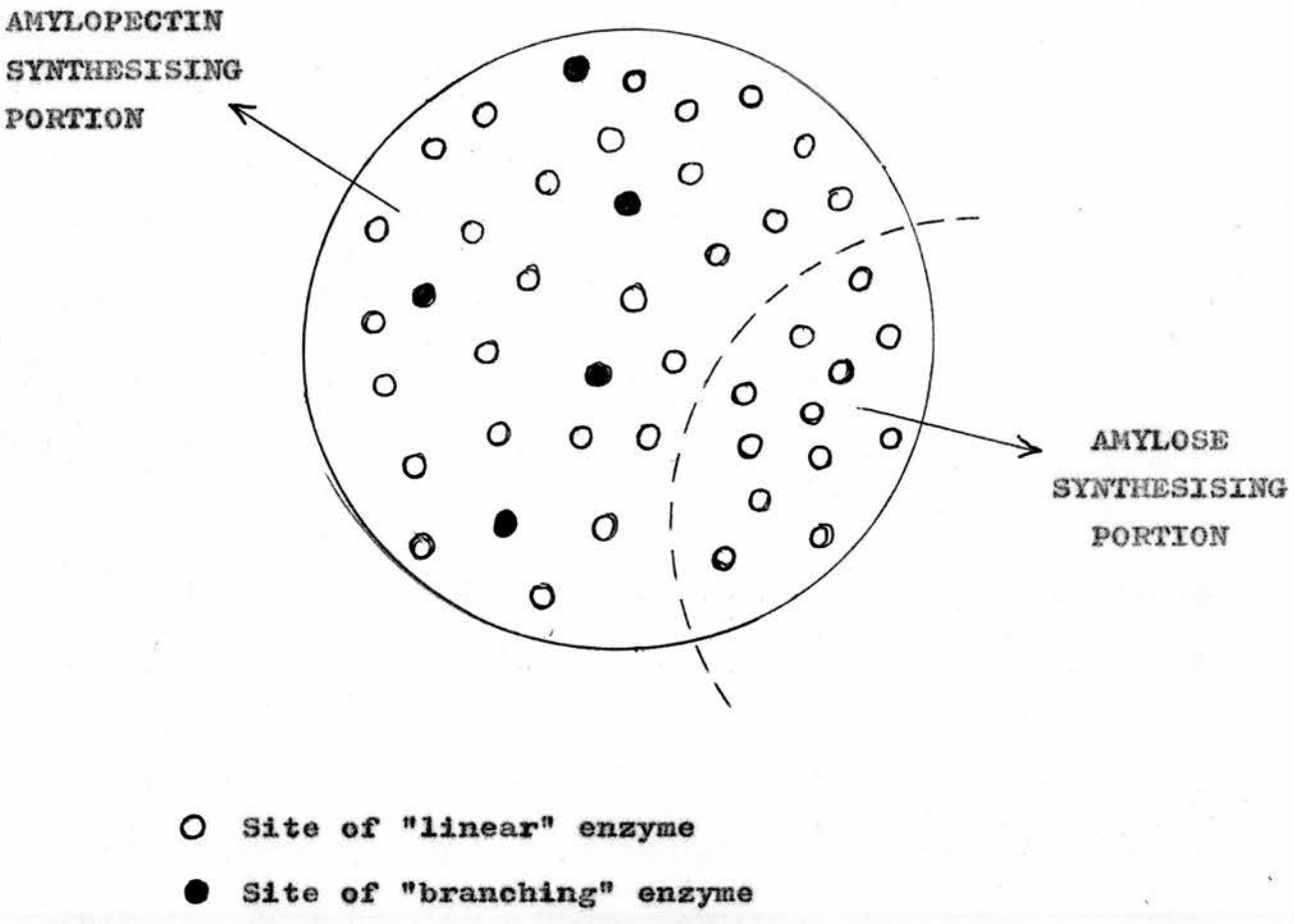


FIGURE 23 PORTION OF GRANULE SURFACE

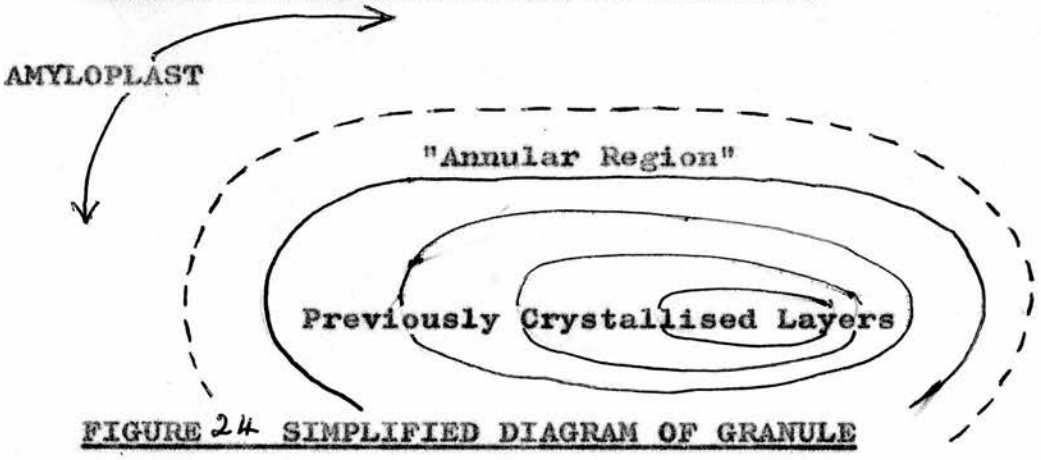


FIGURE 24 SIMPLIFIED DIAGRAM OF GRANULE

percentage amylose content of the granule, the D.P. of the amylose, the chain length and molecular weight of the amylopectin can all be explained if an increasing ratio of linear to branching enzymes are absorbed from the amyloplast. This seems more likely than the increase in phosphorylase activity which must be involved to explain the increase in molecular weight and chain length of the amylopectin, if the biosynthesis is to be explained by the Whelan (1963) theory. The former suggestion is not unreasonable when it is remembered that the amylose synthetase enzyme is bound very tightly to the granule surface (Leloir et al (1957)) whereas Q-enzyme can be extracted from the granule by fairly conventional techniques (Haworth et al (1944)). This would suggest that the "linear" enzyme has a high surface energy of activation for the starch granule whereas the "branching" enzyme has a relatively low surface energy. If this is true the ratio of "linear" to "branching" enzyme adsorbed will increase with the increase in surface area as the granular size increases. This obviously fits the experimental data presented in the previous section.

In conclusion, the present theory accounts qualitatively for the results obtained in biosynthetic experiments from several sources. It is based on the one assumption that Q-enzyme is relatively weakly bound to the granule surface.

SECTION V.

STUDIES ON GLYCOGEN AND ITS EXTRACTION.

SECTION V.

STUDIES ON GLYCOGEN AND ITS EXTRACTION.

INTRODUCTION.

Glycogen, the main reserve carbohydrate of animals, is found distributed throughout the tissues of the body. Numerous methods have been employed for extraction of glycogen from tissues. These include the classical technique of extraction in boiling concentrated alkali (Bernard, (1857), Pflüger, (1904)) as well as cold water (Lazarow, (1942), Drochmans (1963), Bueding and Orrell, (1961)) and hot water (Carruthers (1931), Greenwood and Manners, (1957)), cold trichloroacetic acid (Stetten et al (1956)) and hot trichloroacetic acid (Sahyun and Alsberg (1930)), chloral hydrate (Meyer and Jeanloz (1943)), dimethylsulphoxide (Whistler and BeMiller (1962)), a phenol-water mixture (Laskov and Margoliash (1963)), and glycine buffer (Bueding and Orrell (1964a,b)).

Most of the earlier of the above extraction techniques involve relatively drastic conditions which could cause a partial degradation of the polysaccharide. Since the molecular weight spectrum would thereby be altered the investigation of the nature of the glycogen molecule "in vivo" has been severely handicapped. Early reports that the glycogen extracted was independent of the method used (Bell and Young (1934), Bridgman (1942), - for boiling alkali, boiling water, and hot trichloroacetic acid) delayed investigations /

investigations on the effect of extraction procedure on the molecular weight spectrum and properties of the glycogen molecules. However, in 1956, Stetten, Katzen and Stetten showed that glycogen extracted by short exposures to cold trichloroacetic acid has a considerably higher molecular weight than the material prepared by the classical alkali technique. The milder extraction techniques mentioned above, which have been developed after this date, have all been shown to yield higher molecular weight material than the earlier methods.

This present work was initiated in order to investigate quantitatively the products of the milder extraction techniques (using the classical alkali extraction as a control) in order to find which method yields glycogen most representative of that in living tissue.

EXPERIMENTAL PROCEDURES.

Material.

The extractions were performed upon separate sections of the same liver obtained from a well-fed "barley-bullock" and stored in an acetone-solid carbon dioxide mixture within five minutes of death, in order to reduce any enzymic activity to the minimum. Death was caused by the destruction of the brain tissue by rivet-gun (Edinburgh Municipal Slaughter House, September, 1964).

Extraction Methods.

Alkaline preparation (Pflüger (1904)

A portion of the liver was defrosted, minced and then boiled with /

with 30% potassium hydroxide for 3 hours upon a boiling water bath. After cooling, removal of the soap layer, and centrifugation the precipitate was re-extracted with alkali and combined with the original supernatant. After filtration through glass-wool the glycogen was precipitated with 3-4 volumes of ethanol.

The glycogen obtained by this method is hereafter called OH-glycogen.

Trichloroacetic acid (TCA) preparation (Sahyun and Alsberg (1930), Stetten et al (1956)).

After defrosting a portion of the liver, it was minced and then treated with 10% TCA at 0°-2°C in a high-speed macerator for 2-3 minutes. After rapid filtration through muslin the glycogen was immediately precipitated by the addition of 3-4 volumes of ice-cold ethanol. The residue in the muslin was re-extracted with 5% TCA and its glycogen also precipitated. The glycogen (TCA-glycogen) was then collected by centrifugation at 0°-2°.

Dimethylsulphoxide (DMSO) preparation (Whistler and BeMiller, (1962)).

A portion of the defrosted liver was macerated in DMSO for two minutes. After centrifugation and rapid filtration the glycogen was precipitated by addition of the supernatant to methanol containing ca. 0.2% sodium chloride. The original precipitate was re-extracted and its product combined with the glycogen obtained above.

During the purification of this product (see below) any residual /

residual protein was removed by gentle shaking of an aqueous glycogen suspension with 0.25 volumes of n-amyl alcohol over a period of 12 hours.

The product is identified as DMSO-glycogen.

Phenol-water preparation (Laskov and Margoliash (1963)).

This is based on the phenol extraction method of Kirby (1956) for the preparation of ribonucleic acid. Its main advantage lies in the stability of the polysaccharide to conditions of preparation which immediately destroy the enzymes degrading glycogen.

A portion of the frozen tissue was ground to a coarse powder under liquid nitrogen. This powder was homogenised in a cooled ($4^{\circ}C$) high-speed blendor with 3 volumes of water and 3 volumes of 90% phenol for 4 minutes. The mixture was then gently stirred for one hour at $20^{\circ}C$. After centrifugation the milky upper phase was separated and the lower phase re-extracted. After combination of the products the glycogen was precipitated by 3 volumes of ethanol, centrifuged, dissolved in a minimal volume of distilled water and dialysed for 24 hours against 4 changes of distilled water. The RNA was removed by vigorously mixing with the solution, 1 volume of 2-methoxyethanol, 1 volume of 2.5 M dipotassium hydrogen phosphate and 0.05 volumes of 33.3% orthophosphoric acid. The RNA was then centrifuged off. The Ph/H_2O-glycogen was then obtained by precipitation of the milky, washed, lower phase with 4 volumes of ethanol.

Purification /

Purification of the Glycogens.

Purification of all 4 glycogens was achieved in basically the same manner, by repeated re-dissolving of the glycogen precipitate and low speed centrifugation of the solution; then precipitation of the glycogen with ethanol followed by high-speed centrifugation and discarding of the supernatant. Care was taken with the centrifugations since prolonged high-speed centrifugation is necessary to ensure a quantitative yield of the lower molecular weight components. Similarly, if the low speed centrifugation is performed at an excessive speed or for too long a time, a preferential loss of the higher molecular weight components can occur. These points have also been noted by Orrell, Bueding, and Reissig (1964).

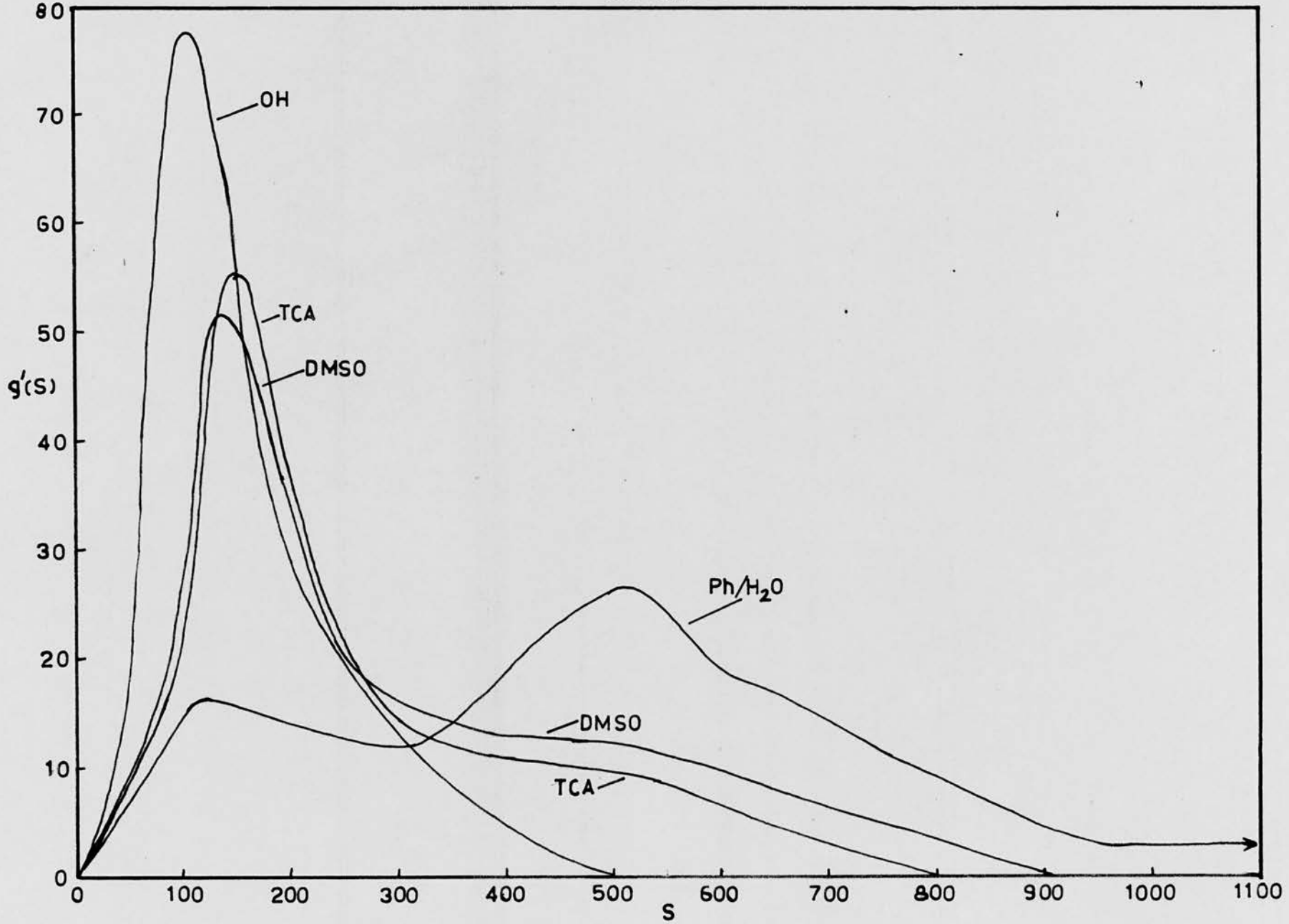
The glycogens were considered pure when a trace of electrolyte was required to induce flocculation. In all cases care was taken to add a distinct excess of alcohol.

All the glycogens were treated in exactly the same manner with the exception of the OH-glycogen which was precipitated twice with 4 volumes of glacial acetic acid (Pflüger's method) before the alcohol precipitations, and the DMSO-glycogen in which the solvent initially used was decreasing amounts of DMSO until the glycogen was almost colourless, before the normal water-alcohol purification.

Yields were of the order of 1% of wet tissue weight.

Measurement /

FIGURE 25



EFFECT of EXTRACTION METHOD upon GLYCOGEN DISTRIBUTION.

Measurement of Distributions.

Samples of the purified glycogens were dissolved in 0.1 M sodium chloride solution (ca. 10-12 mg./ml.). Sedimentation coefficient distributions were determined with the Spinco model E analytical ultracentrifuge using double-sector cells at approximately 9300 r.p.m. The temperature was controlled at 20°C. The photographic plates were measured on a two-dimensional travelling microscope reading to 0.001 cm. These results were converted to sedimentation coefficients by the method of Baldwin (1954) as detailed by Bryce et al (1958), where a full description of the relevant theory is given. All tedious calculations in the present work were programmed for the Atlas Autocode Computer (Manchester University). All curves are normalised, and corrected for diffusion.

Results.

The resultant sedimentation distribution curves for the glycogens obtained by the four extraction-methods on the same sample of bullock liver are illustrated in figure 25. In agreement with earlier results, it is evident that the alkali-extracted sample contains both the smallest breadth of distribution and the smallest molecular weight material (S max = 100). The TCA- and DMSO- extractions yield very similar products (S max = 160), with the DMSO-extraction having a greater proportion of higher molecular weight /

weight material; in both samples there is evidence of polydispersity, i.e. containing more than one component (Bryce et al (1958).

The phenol/water extraction product exhibits extreme polydispersity and shows two distinct peaks (S max = 110, 510): Further, the amount of the high molecular weight material is very much in excess of that of the lower.

By using an extraction method based on the cold-water extraction method of Lazarow (1943) as modified by Drochmans (1963), Orrell and Bueding (1964b) obtained a product similar to that yielded by the phenol/water extraction method used here. However there was a practically total loss of the lower molecular weight material. They have ascribed this loss of the lighter peak to insufficient high speed centrifugation of the glycogen solution after precipitation with alcohol. To avoid any similar loss of material in the present work, great care was taken in the centrifugations; all the glycogens were centrifuged for the same time and at the same force-field. At worst, therefore, a relative comparison of the products will be accurate.

Another point which also suggests that the distribution of phenol/water-glycogen is not caused by differential sedimentation losses is that the percentage yield of product is essentially the same as that for the KOH extraction (and roughly double that found for the DMSO- and TCA- extractions). In contrast Orrell and Bueding point out (1964b) that the Lazarow-Drochmans procedure results /

results in a recovery of less than 20% of the glycogen originally present in the tissue and that occasionally less than 4% is recovered.

It would appear, therefore, that the phenol/water extraction method is the most efficient of the 4 methods examined, and from the rather sparse quantitative data in the literature this method probably yields a sample most representative of "native" glycogen.

Effect of Degradation upon the Glycogen Distributions.

The glycogens extracted by the methods outlined above were subjected to the action of various degrading reagents. In an attempt to gain some insight into fine molecular architecture, the changes in distribution caused by the degradation were measured. The various reagents used and their effects are described below and in figures 26-30.

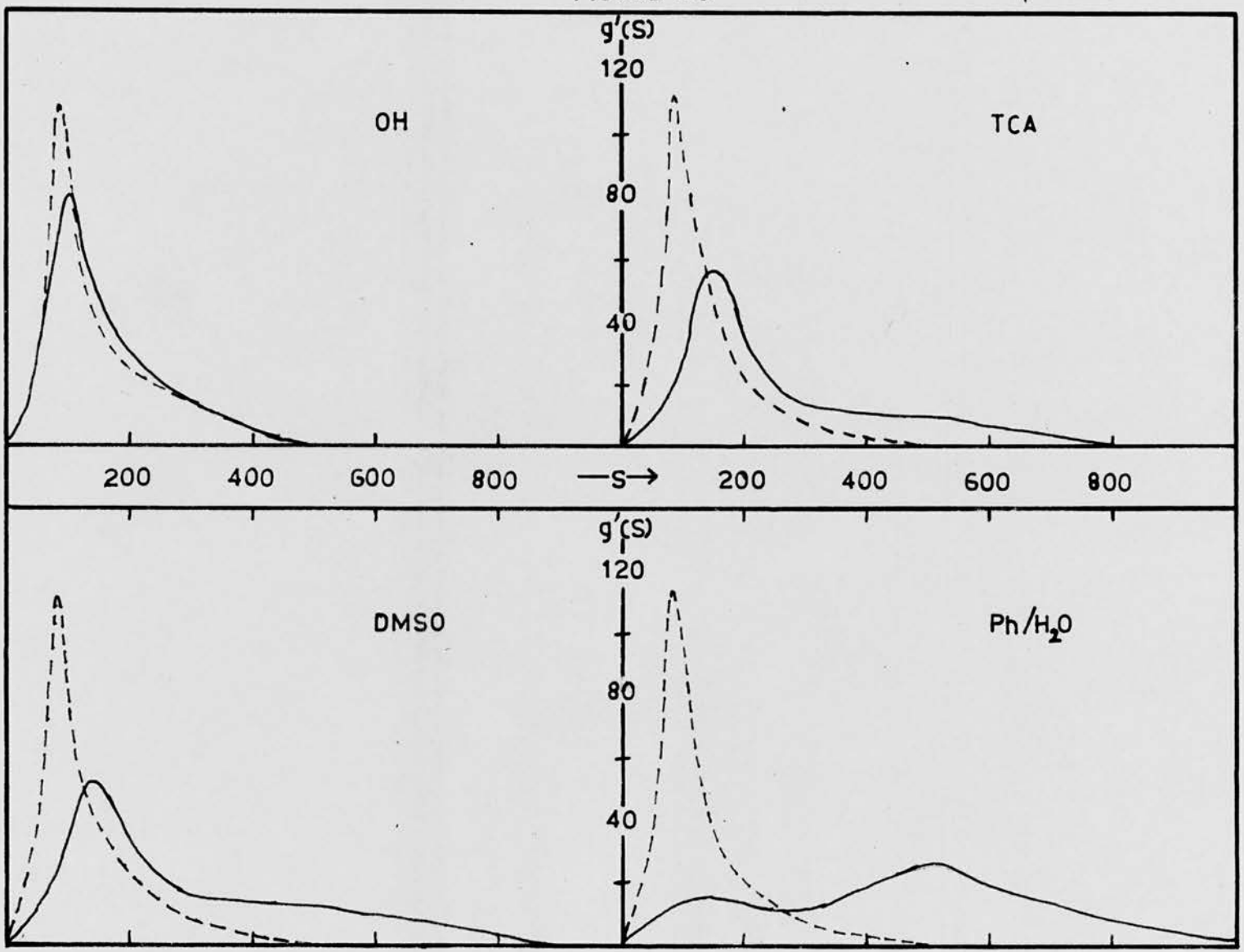
Hot, concentrated alkali:- The effect of 30% potassium hydroxide at 100°C upon glycogens has been shown to be (Stetten and Katzen (1961)) the production of a polydisperse series of relatively stable polysaccharide acids, corresponding to the glycogen isolated by the classical Pflüger method, and small amounts of free isosaccharinic acids. The apparent alkali-stability of glycogen after preliminary exposure to alkali is due to replacement of the reducing end group of each molecule by a saccharinic acid residue. The effect of this alkali treatment upon the distributions obtained in the present work is shown in figure 26 . It can be seen that all the glycogens degrade to the same product (within experimental /

experimental error), and that this product is very closely related to that obtained directly by the OH-extraction method. The slight change in the distribution of the OH-glycogen material on retreatment with alkali probably results from the fact that the conditions were not strictly anaerobic; under oxidising conditions alkali-sensitive linkages may be attacked other than those adjacent to terminal aldehyde groups. (Stetten and Katzen, (1961)).

Hot water:- The effect on the distributions of the three more disperse glycogens caused by water at 100°C under atmospheric conditions or with oxygen-free nitrogen bubbling through the solution is illustrated in figure 27 . It can be seen that the degradation is practically totally independent of the presence of oxygen, unlike the dilute alkali degradation (see below). The degradative effect is very marked for the higher molecular weight species. This is shown very markedly in the case of the phenol/water extracted glycogen, where degradation causes total loss of material above 640S, whilst in the original glycogen the region above 640S had contained ca. 20% of the molecular species.

Hot, dilute alkali:- Stetten and Katzen (1961) and Whistler and BeMiller (1958) have shown that, in hot dilute alkali - apart from the reaction which occurs with concentrated alkali - the enhanced solubility of oxygen in the solution induces another reaction. This reaction, which is rapid only under oxygen, is probably one of oxidation followed by random splitting of the large polysaccharide molecule. This latter reaction may be eliminated /

FIGURE 26



EFFECT of 30% KOH (100°C, X3hours) upon the various GLYCOGENS.

ORIGINAL—— DEGRADED - - - - -

FIGURE 27

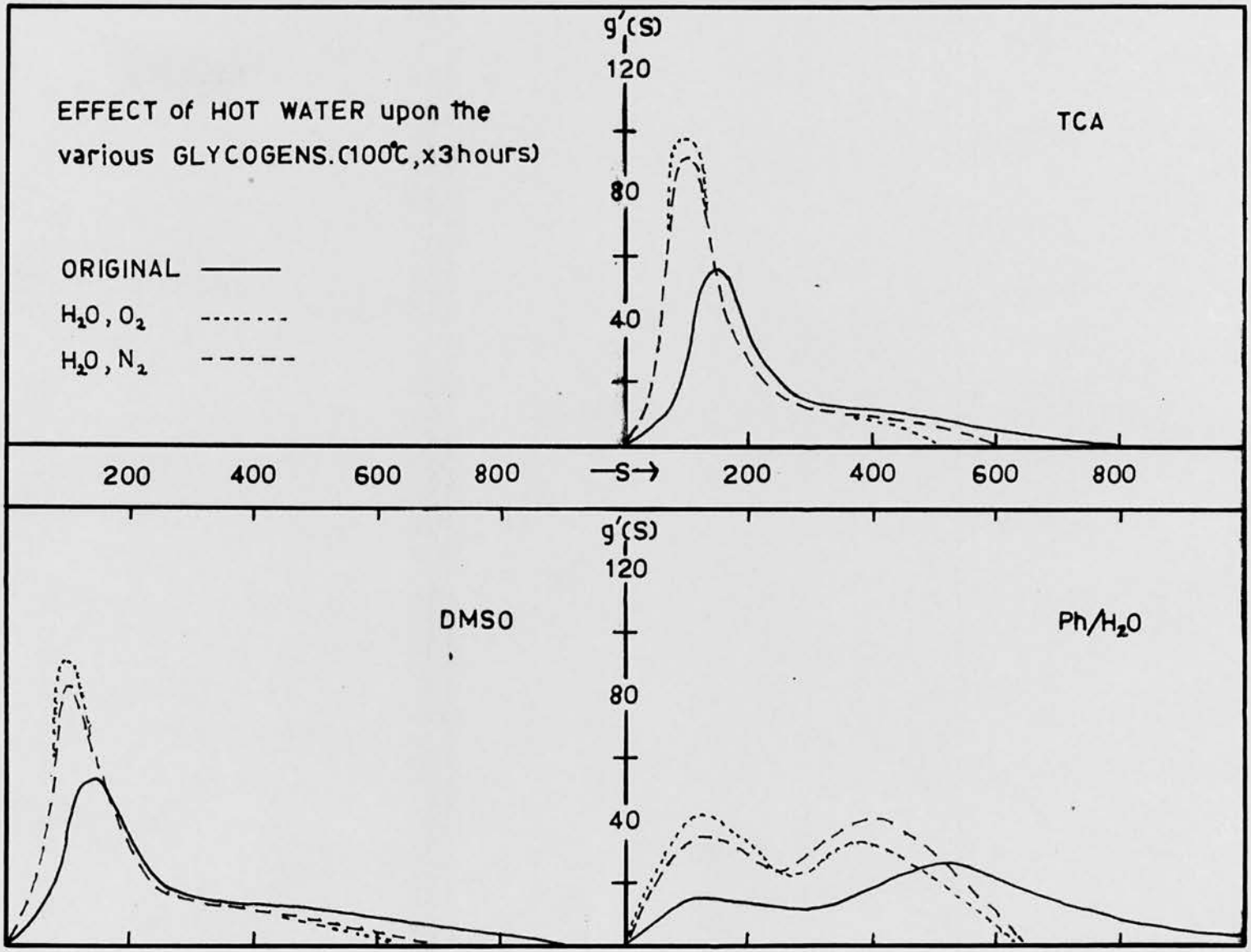
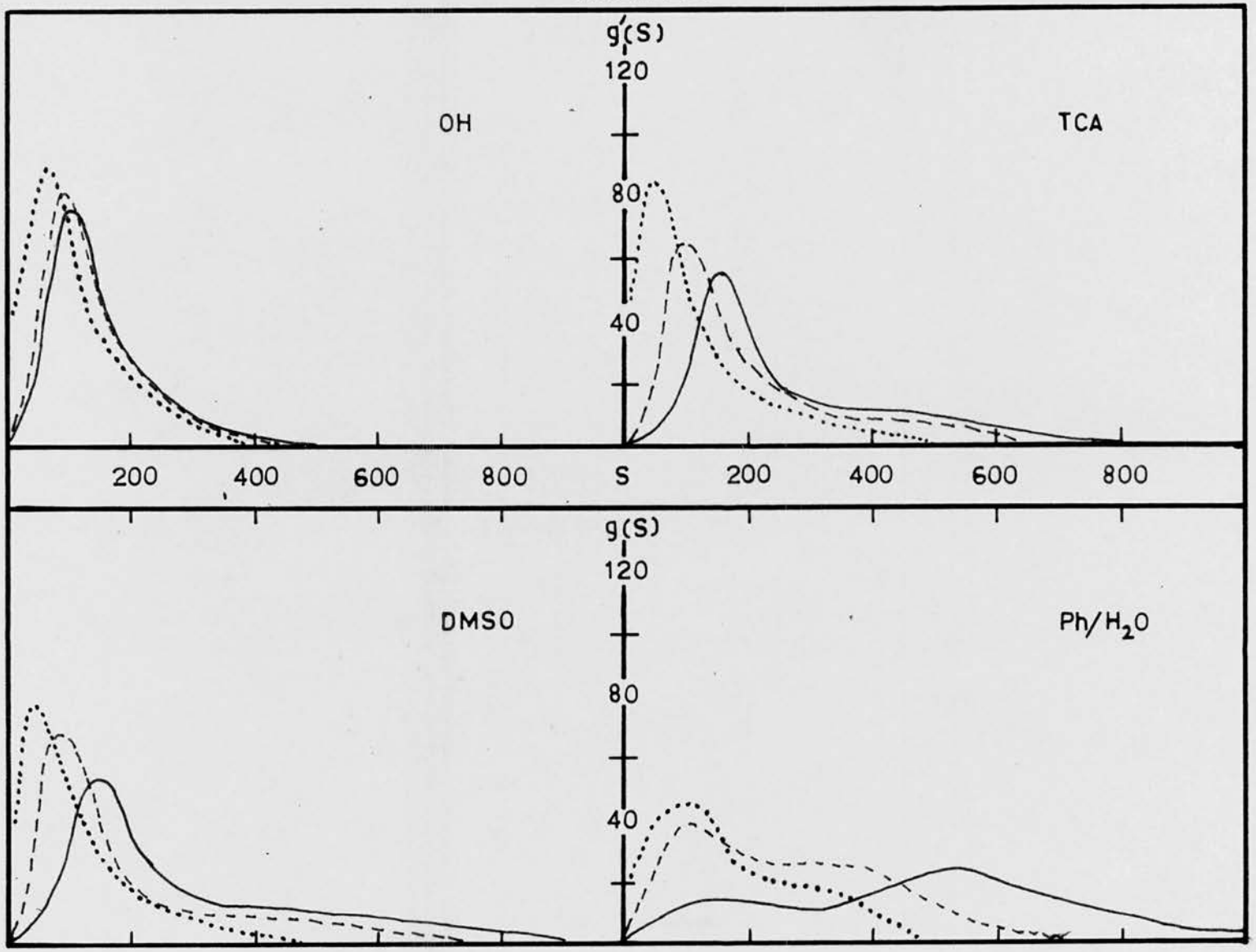

EFFECT of HOT WATER upon the various GLYCOGENS. (100°C, x3 hours)
ORIGINAL
H2O, O2
H2O, N2
g'(S)
120
80
40
TCA
200
400
600
800
—S→
DMSO
Ph/H2O

FIGURE 28



EFFECT of 0·1 M KOH (100°C, X3hours) upon the various GLYCOGENS.

ORIGINAL —— UNDER N_2 - - - - UNDER O_2 ·····

FIGURE 29

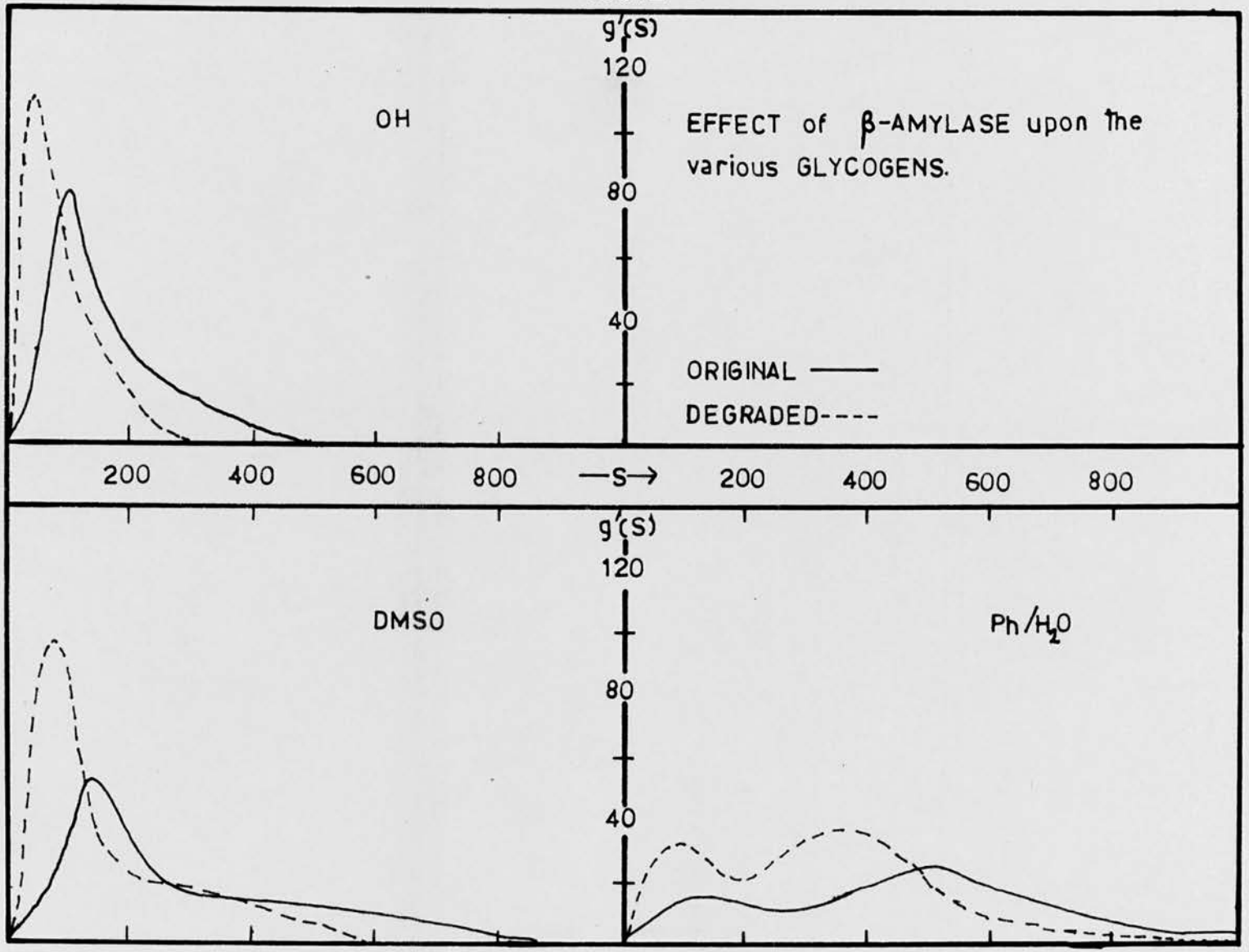
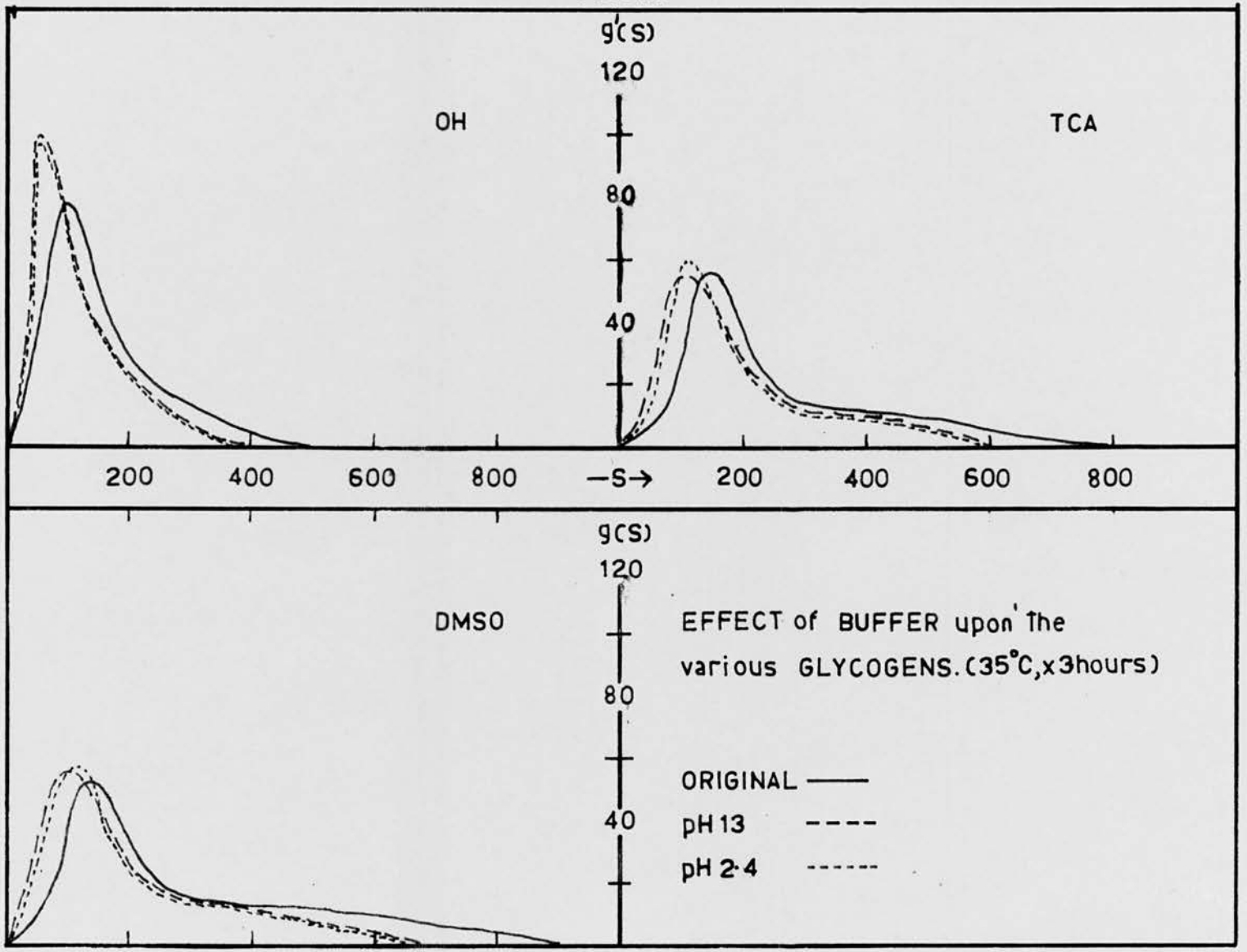

g'(S)
120
80
40
OH
EFFECT of β-AMYLASE upon the various GLYCOGENS.
ORIGINAL
DEGRADED----
200
400
600
800
—S→
200
400
600
800
g'(S)
120
80
40
DMSO
Ph/H2O

FIGURE 30


g(S)
120
80
40
OH
TCA
200
400
600
800
—S→
200
400
600
800
g(S)
120
80
40
DMSO
EFFECT of BUFFER upon the various GLYCOGENS. (35°C, x 3 hours)
ORIGINAL
pH 13
pH 2·4

eliminated by carrying out the reaction in an oxygen-free atmosphere, and a comparison of the two reactions is shown in figure 28. It is clear that the degradation occurring under nitrogen is merely a milder form of that in concentrated alkali, where the oxygen solubility is extremely low. Hence the distributions should be intermediate forms between the original material and that degraded (figure 26) by concentrated alkali.

Treatment of OH-glycogen with dilute alkali in an oxygen-containing atmosphere, causes a distinct change in the distribution, illustrating the independence of the two alkali-induced polysaccharide-degrading reactions.

Detergents and other hydrogen-bond breaking reagents:- It has been suggested that very high molecular weights in glycogen might be the result of either (i) protein-bound aggregation (Lazarow(1942)), or (ii) a hydrogen-bonded aggregate as is found in the proteins. The work of Orrell et al (1964) indicates that (i) may be unlikely as the glycogen distributions are unaffected by conditions which would denature a protein. In addition to this, the extremely high molecular weights in the phenol/water extracted glycogen are obtained from an aqueous layer, from which protein has been practically quantitatively extracted (Kirby (1956)). However, none of the evidence is as yet fully conclusive, since if the protein was chemically bonded to the glycogen molecules, its aggregative effect would still remain after the configurational change caused by denaturation.

It /

It is easier to check the possibility of hydrogen-bonding aggregation between the polysaccharide chains themselves. Orrell et al (1964) have shown that prolonged incubation with various reagents known to rupture hydrogen bonds, such as 8M urea, 8M guanidine, 2M thiocyanate, 8M lithium bromide and non-ionic, anionic, and cationic detergents (1%), result in no changes in the molecular weight distributions. This has been confirmed qualitatively with the samples used in the present work; the sedimentation patterns in water and 8M urea were not radically different. Also repeated precipitations with ethanol and drying did not have any effect upon the distribution.

β-amylase:- The effect of the enzyme β-amylase upon the glycogen distributions is illustrated in figure 29 .

β-amylase degrades starch-type polysaccharides with the production of maltose. Degradation occurs from the non-reducing end-groups and the enzyme appears to be completely specific for α-1→4 bonds. Its attack upon the branched glycogen is halted when the outer chains have been degraded to within two or three glucose units of the 1→6 branch point. The β-amylolysis limits of the four glycogen samples are shown below:

	GLYCOGEN			
β-limit*	OH	TCA	DMSO	Ph/H_2O
	42%	42%	39%	38%

* Digests were performed using excess crystalline β-amylase (free from α-amylase) over 24 hours at 35°C. β-limits were the average of two independent estimations.

Experimental error is of the order of ± 1-2% so that the samples do not show any great differences. There is a slight trend for higher molecular weight material to have a lower β-limit.

These results will be discussed quantitatively below, where it will be shown that all the molecular sizes have been degraded to the same extent.

Buffer Solutions. Orrell et al (1964) have reported that exposure of glycogen to pH values greater than 13 or less than 3.5 caused irreversible degradation. Accordingly three of the glycogens were dissolved in buffer (pH 2.4 and 13) and allowed to stand for three hours at 35°C. The resultant effects upon the distributions are shown in figures 30 .

The similarities of the degradations from a molecular-distribution viewpoint is quite marked. The distributions of the degraded OH-glycogen are very similar to the distribution of the OH-glycogen β-limit dextrin which would indicate that all the molecular weight species were degraded to approximately the same extent. There is however a marked molecular-weight "tail". The distributions of the degraded TCA- and DMSO- glycogens indicate that the changes in the very high molecular-weight material are much more marked than in the corresponding lower molecular-weight species.

CALCULATION /

CALCULATION OF MOLECULAR-WEIGHT DISTRIBUTION CURVES.

Molecular-weight distribution curves can be obtained from sedimentation coefficient distributions if the relations S = f(c) and S = f(m) are known. (Bryce et al (1958), Baldwin (1954)). There is some discrepancy in the literature as to the quantitative nature of the s-c relationship. Bridgeman (1942) and Madsen and Cori (1958) both found the sedimentation coefficient, S, to be independent of the glycogen concentration. Bryce et al (1958) have reported a relation of the type $S_{20} = (S_{20})_0 (1-kc)$ and Larner et al (1956) used a relationship of the type $S_{20} = (S_{20})_0 - k.S_{20}^2.c^2$ In view of these obvious discrepancies, a glycogen was fractionated (see below) and its concentration dependence measured. It was found that over the range 450S - 1200S the sedimentation coefficient was independent of the concentration (within experimental error). By using this relation and that of Bryce et al (1958) for the molecular weight dependence - $ds/dm = (4,39 \times 10^{-25})S^{-0.59}$ - molecular weight distribution curves were calculated for all four samples and are shown in figure 31 . The extreme heterogeneity of the Ph/H_2O-glycogen is again emphasised as are the extremely skew distributions of the DMSO- and TCA- glycogens.

Accuracy.

Random experimental error is difficult to assess for such a procedure. The largest error is probably introduced by the initial measurements of the photographic plates. The outline of the /

the Schlieren pattern is of finite thickness and is not sharply defined. In the present work the two extremes of the Schlieren pattern were measured and their average taken. The base-line, also present in the photograph because double-sector cells were used, was measured by the same technique. Hence although the absolute accuracy of the travelling microscope is $\pm$0.001 cm. the absolute heights of the Schlieren patterns are probably no better than $\pm$0.006 cm. (approx.$\pm$1%).

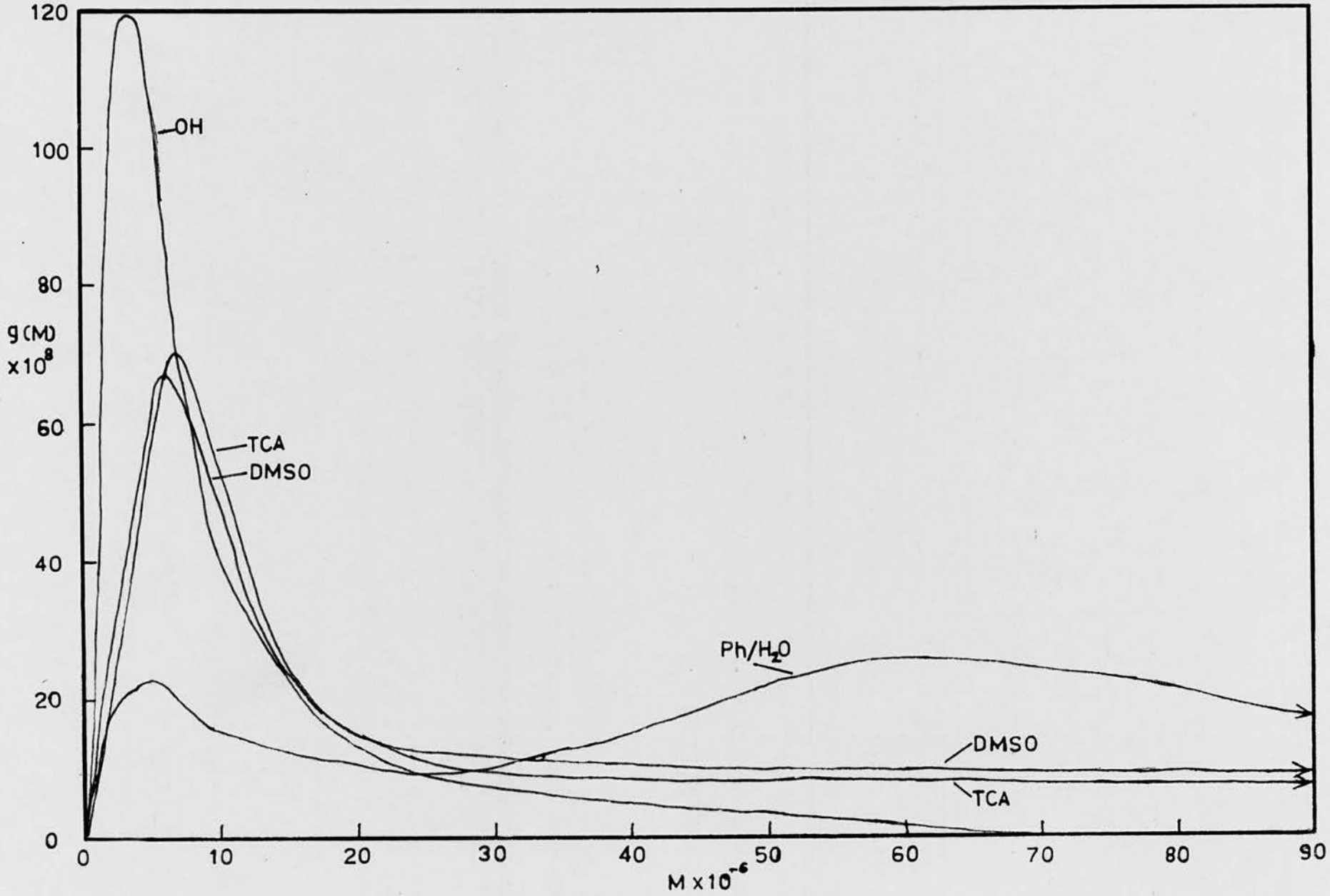
However this error is small compared with the systematic errors introduced by the form of the relations of sedimentation coefficient with concentration, and with molecular weight. The accuracy of measurement of sedimentation coefficient is ca. 2%. Also Bryce et al (1958) have indicated that the S-M relationship is not necessarily extremely accurate but is as good as can be expected. (Probably $\pm$5%).

In conclusion, it is probable that these distributions are not accurate absolutely since the form of the curve, especially at high molecular weights is very dependent upon the S-M and S-C relations. However it is thought that the relative values for all the samples will be of the correct order.

EFFECT OF β-AMYLOLYSIS.

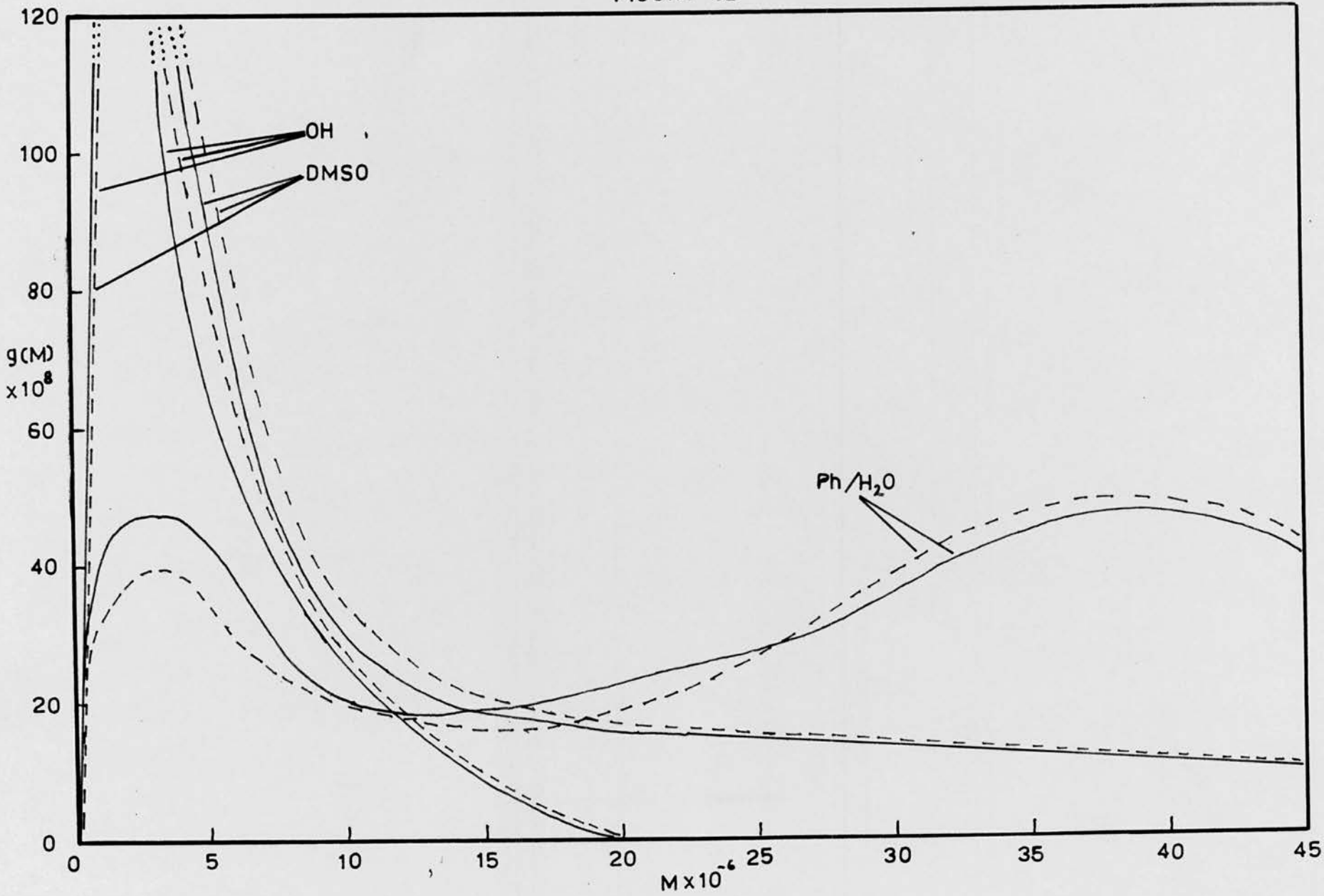
Although the general course of the reaction of β-amylolysis is well-established there have been no quantitative reports on the effect of the enzyme upon very broad molecular-weight distributions such as were found in the present work. It seems possible that the /

FIGURE 31



MOLECULAR WEIGHT DISTRIBUTION CURVES for the GLYCOGENS.

FIGURE 32



MOLECULAR WEIGHT DISTRIBUTION CURVES for the GLYCOGEN β-LIMIT DEXTRINS.
EXPERIMENTAL (——) and THEORETICAL (-----).

the β-amylolysis may be greater for larger molecular weights. Bryce et al (1958) have shown that, over the very limited range of molecular weights used in their work ($1x10^6$ to $13x10^6$), there was no appreciable preferential and mere extensive degradation of material of either low or high molecular weight. It was therefore considered important to extend this work to the very large molecular weight ranges obtained here.

A curve for the various β-limit dextrins was calculated assuming that all the molecules were degraded to the same extent (i.e. to their β-limit - see Section 5c). This was achieved by dividing the original glycogen molecular-weight distributions (figure 31) into a series of lamellae of discrete molecular weight and treating each as a homogeneous polymer. If the attack of the β-amylase is random with respect to molecular size, then the decrease in molecular weight during β-amylolysis will be proportional to the number of non-reducing end-groups i.e. to the molecular weight, and the molecular-weight distribution for the limit dextrin (M_{LD}) will be simply related to that of the original (M_0) by

$$M_{LD} = M_0(100-\beta)/100$$

where β = β-amylolysis limit

and normalisation of the corresponding curve is achieved by

$$g(M_{LD}) = g(M_0)100\ /(100-\beta).$$

The results of such a calculation are compared with the experimental measurements made directly upon the β-limit dextrins in figure 32 . Agreement is satisfactory and within experimental error.

Conclusion /

Conclusion.

It appears, therefore, that glycogen is degraded by β-amylase to the same extent, regardless of its molecular size, over a very wide range. High molecular-weight glycogen has no appreciable structural differences from the low molecular-weight material. These results complement those of Laskov and Margoliash (1963) who found that the percentage of non-reducing end-groups in glycogen did not vary over the range of molecular weights 20 -350 x 10^6.

FRACTIONATION.

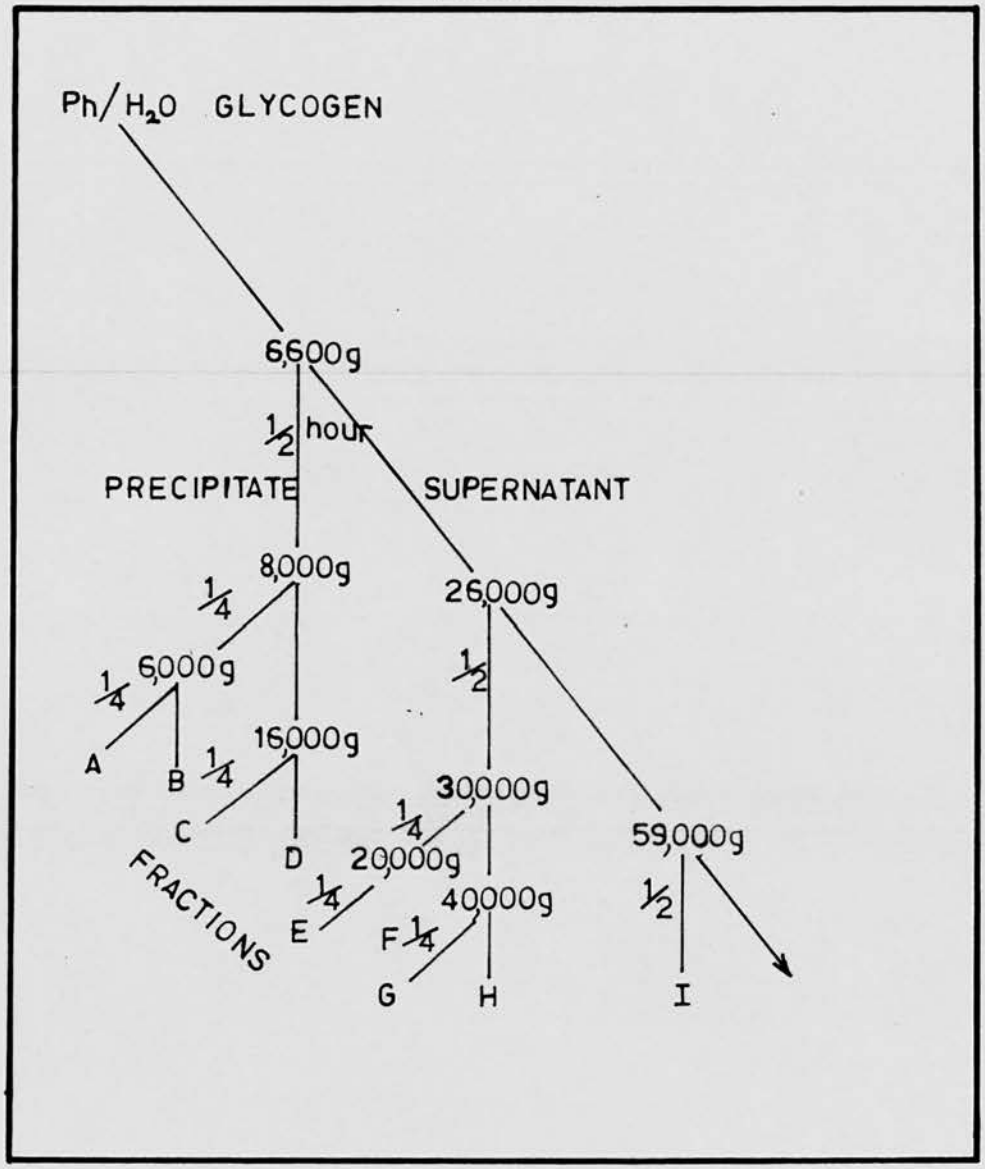
A portion of the phenol/water glycogen was fractionated by differential sedimentation in 0.1M sodium chloride solution on a Spinco Model L ultracentrifuge in order to provide fractions from which the sedimentation coefficient-concentration relationship could be accurately established. The scheme used is illustrated in figure 33. Three fractions were picked because of their size and concentration. Sedimentation coefficients were measured in 0.1M sodium chloride at 9340 r.p.m. on the Spinco Model E analytical ultracentrifuge. The temperature was controlled at $20^{\circ}C \pm 0.05^{\circ}C$.

The results are shown in figure 34. It is clear that any concentration dependence is within the experimental error.

DISCUSSION.

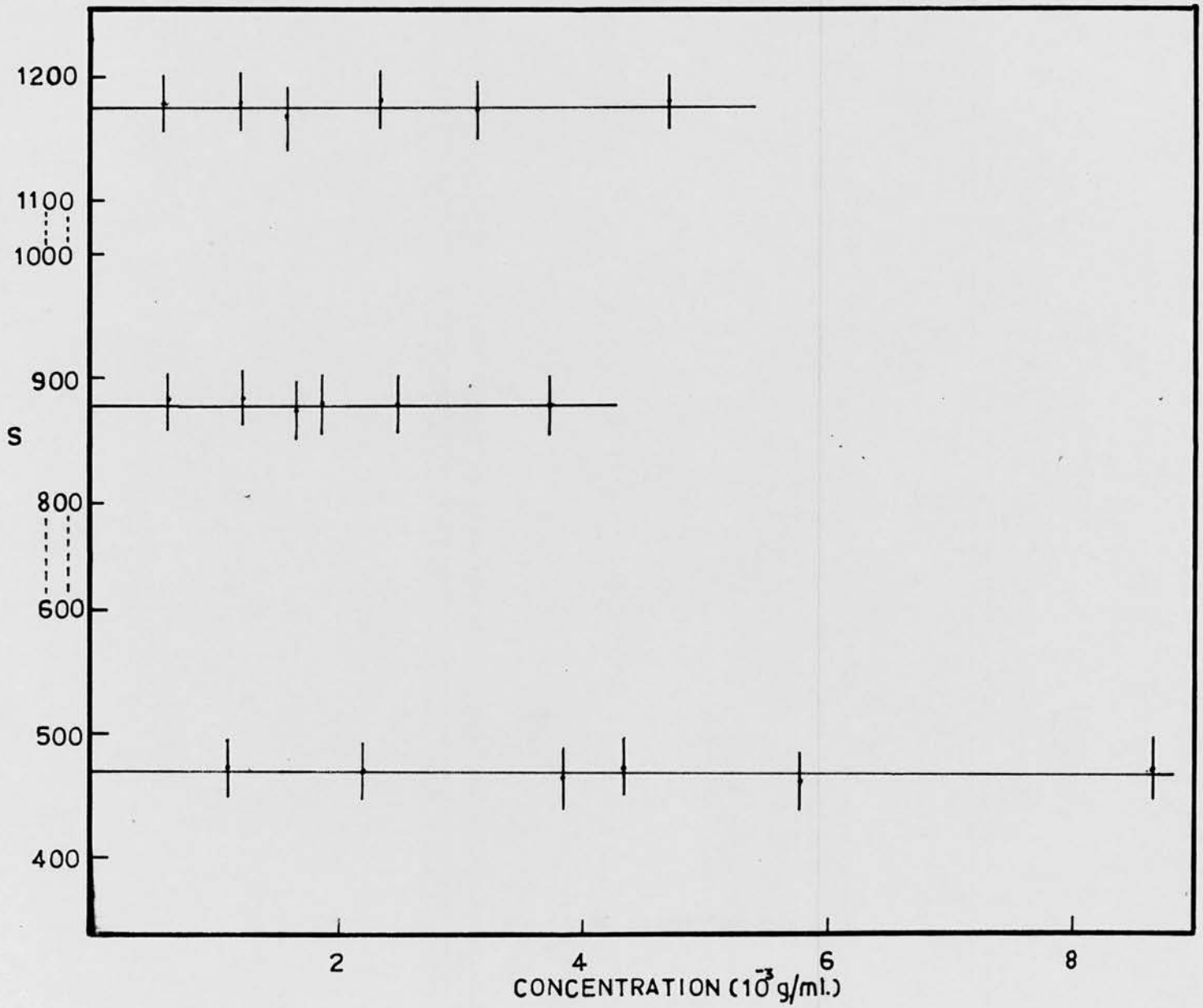
It is clear from the sedimentation and molecular-weight distributions that there are appreciable amounts of material of molecular weight of 200-300 x 10^6 in the case of the DMSO- and TCA-glycogens and of 4-500 x 10^6 in the case of the phenol/water sample. No /

FIGURE 33



GLYCOGEN FRACTIONATION SCHEME.

FIGURE 34



S versus C for GLYCOGEN FRACTIONS in 0·1M NaCl.

No attempt has yet been made to explain the apparent inconsistency of the theoretical calculations of maximum molecular size as determined by Pollard (1958) (see also Madsen and Cori (1958) and Stetten and Katzen (1961)) and the much greater molecular sizes obtained experimentally.

Theoretical Molecular Size of Glycogen.

If the glycogen molecule is assumed with an average of 4 α-1→4 linked glycosyl units between each 1→6 link and an average of 8 α-1→4 linked residues in the outer chain (Larner et al (1952)), then the model structure illustrated in figure 35 is obtained. When successive tiers are added peripherally to this model, doubling the number of non-reducing termini with each additional tier, a limit of size is reached. The existence of such a limit results from the fact that space available for further branching and growth at the periphery of the growing glycogen molecule is rapidly used up.

i.e. if t = number of tiers

r = radius of glycosyl residue

R = radius of glycogen molecule

then the total number of glycosyl residues approximately equals $2^{t-1} \times 13$ and

$$R = (6 + 10t)r$$

(Full details of these calculations are given in the above references).

The maximum possible molecular size on these calculations is a 17 tier model and its molecular weight is 138×10^6. The radius of /

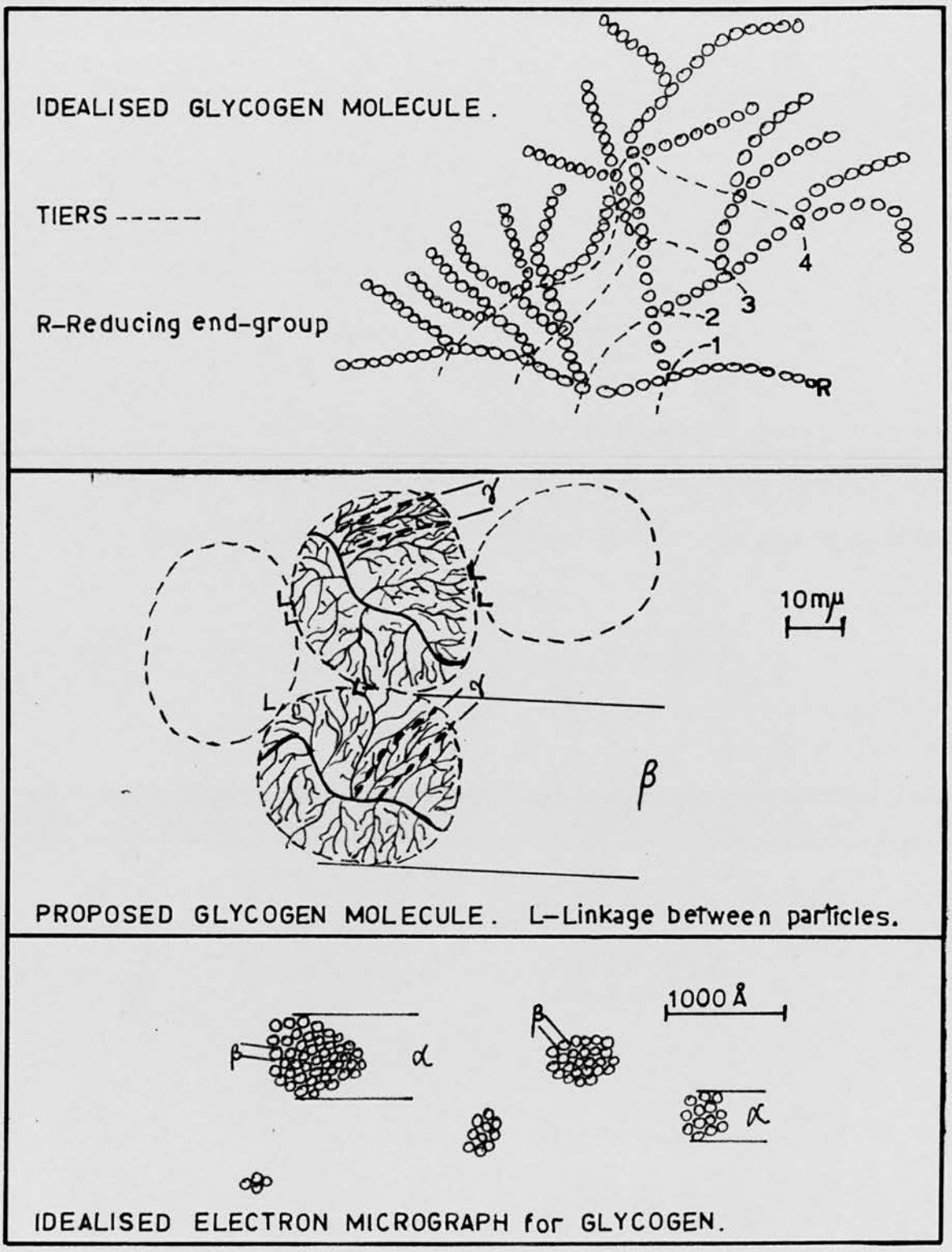
of the particle (assuming the radius of the glycosyl residue to be of the order of 2Å) would be 35 mμ. This would appear to be irreconcilable with the actual experimental molecular weights.

However the situation is clarified when the recently published electron micrographs of Drochmans (1963) and Orrell, Bueding, and Reissig (1964) are considered. Both of these studies were made utilising glycogen with an appreciable amount of high molecular weight material. An idealised drawing of an electron micrograph is shown in figure 35. Using the nomenclature of Drochmans (1963) the glycogen molecules, as viewed microscopically, consist basically of units 60-200 mμ in diameter, called α-particles. Within these particles, smaller units 20-40 mμ in diameter may be recognised and have been called β-particles. Drochmans also identifies a finer structure (γ-elements) consisting of rods (3mμ x 20 mμ) and claims that the β-particle results from the regular deposition in two perpendicular planes of these rods.

It is clear that the β-particles are of the same order, though smaller, than the maximum size of a theoretical glycogen molecule. From a theoretical point of view, particles 20-40 mμ in diameter would have 5-12 tiers and molecular weights of $0.03\text{-}4 \times 10^6$. Orrell, Bueding and Reissig (1964) estimated the size of the β-particle in their micrographs to be of the order of $2\text{-}3 \times 10^6$ (with a large error). This would ideally correspond to a molecule having a diameter of 46 mμ.

The above evidence suggests that the large glycogen molecules are composed of a series of sub units (β-particles) each with a maximum /

FIGURE 35


IDEALISED GLYCOGEN MOLECULE.
TIERS -----
R-Reducing end-group
1
2
3
4
R
10mμ
L
PROPOSED GLYCOGEN MOLECULE. L-Linkage between particles.
1000 Å
IDEALISED ELECTRON MICROGRAPH for GLYCOGEN.

maximum size of 11-12 tiers. The molecule is probably limited to this number of tiers by the steric requirements of the synthesising enzyme. It would not be expected that an enzyme could synthesise such a crowded molecule as a 17-tier glycogen since its own size will be appreciable (see e.g. the enzyme α-amylase, figure 41a).

Further evidence for the unusual nature of large molecular weight glycogen is provided by the extremely high apparent reducing values of glycogens containing appreciable amounts of the large molecular weight species.

GLYCOGEN

	TCA	DMSO	Ph/H_2O
Reducing end-group	2.5%	2.0%	4.6%

The ideal model structure for glycogen (see figure 35) would contain only one reducing end-group per molecule, and the molecular weights evaluated on this basis would be ca. 7000 for the TCA- and DMSO- glycogens and ca. 3000 for the phenol/water glycogen. This obviously cannot be correct, and again the evidence points to the large molecular weight glycogens being composed of a group of smaller sub-units.

The bonding which holds these sub-units together may be one of the following types:

(a) hydrogen bonding between polysaccharide sub-units

(b) bonding of sub-units through a protein molecule

(c) joining of sub-units by one or more chains of α-1 4 linked glycosyl residues.

(a) would appear extremely unlikely for the reasons given previously so /

so a structure based upon (b) or (c) is most likely on the present evidence. This structure is illustrated in figure 35. It is interesting to note that Drochmans' γ-elements can be accounted for as branched segments from the main chain.

The present work cannot confirm unequivocally the proposed "sub-unit hypothesis". All that can be said is that none of the results are inconsistent with this hypothesis. For example, the constancy of the extent of degradation by β-amylase over the very large molecular weight ranges studied would not be expected if the glycogen were a simple tiered structure. Such a structure would become externally more dense as the molecular size increased, and it would be expected that there would be increasing steric hindrance, with correspondingly lower β-limits. Also the evidence from the various degradations suggests that the changes in the high molecular weight species are much greater than would be expected from the simple model, when compared with the corresponding changes in the lower molecular weight species.

Further evidence is provided by the data from the density-gradient ultracentrifugation of the total glycogen samples (Dr. A.R. Procter).

GLYCOGEN

	KOH	TCA	DMSO	Ph/H_2O
Buoyant Density	1.663	1.663	1.662	1.662

This data shows that the large molecular weight material is no more dense in solution than the smaller molecular weight material. This again favours the sub-unit model.

Further /

Further experimentation is obviously required to clarify the nature of large-molecular weight glycogen, e.g. electron micrographs on a fractionation series of a broad molecular weight glycogen. Large molecular weight samples would be expected to be practically exclusively groups of β-sub-units (α-particles) while the low molecular weight species would be expected to be composed of separate β-particles.

SECTION VI.

STUDIES ON AMYLOSE.

SECTION VI.

STUDIES ON AMYLOSE.

INTRODUCTION. Studies of the solution properties of amylose, the basically linear component of starch, presents many difficulties and there are conflicting reports of its behaviour in aqueous solution. Husemann et al (1962), Burchard (1962) and Čeh and Vene (1959) found retrogradation of amylose in aqueous solution, whereas Banks and Greenwood (1963) found no evidence for this in 0.33M potassium chloride solution. The latter workers, from light-scattering, viscosity, and sedimentation measurements on amylose fractions concluded that in this solvent amylose molecules behave as random coils. In contrast, Rao and Foster (1963) have suggested that the conformation of amylose in neutral aqueous solution is that of a stiff coil with an essentially helical backbone contour. Furthermore, Burchard (1963) from light-scattering measurements reports the presence of a second ~~distinct~~ virial coefficient in aqueous amylose solutions which has not been shown by other workers. The importance of using fractions which are linear (as shown by β-amylolysis) has been indicated by Banks (1960) and Banks and Greenwood (1963).

EXPERIMENTAL.

Isolation and Purification of Amyloses: The starch obtained from potato was isolated and purified as described by Greenwood and Thomson (1958). One portion was pretreated with liquid ammonia (Banks et al (1959)). After dispersion to give a 0.5% solution /

solution at 98° under nitrogen the total amylose was removed as the butanol-complex by high-speed centrifugation.

Subfractionation of the Amyloses: (Dr. A.R. Procter). Subfractionation was achieved from dimethylsulphoxide solutions by the method of Everett and Foster (1959). Only linear subfractions were used for light-scattering measurements.

Light-Scattering Technique: As detailed in Section II.

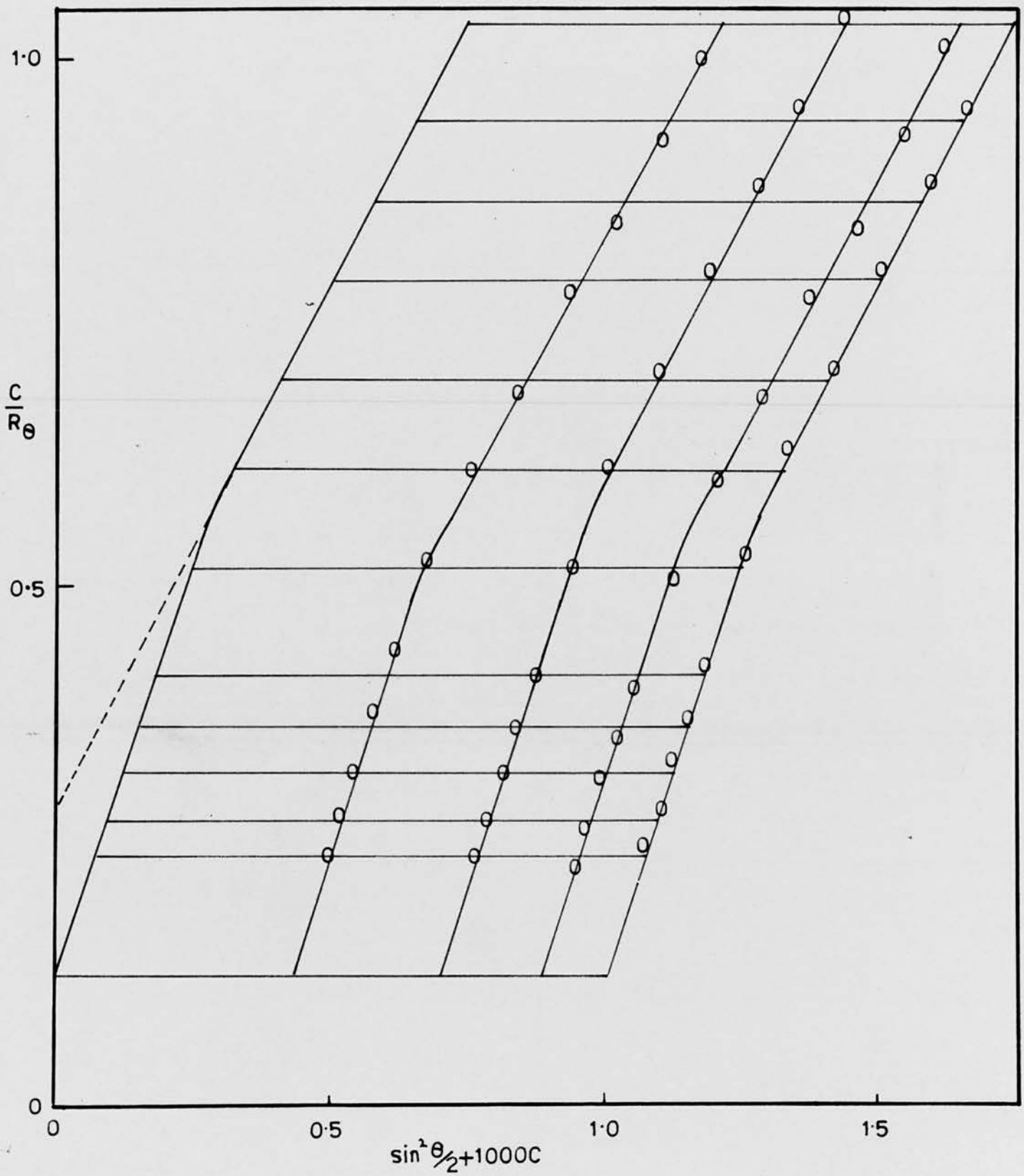
RESULTS.

Zimm-Plots of Total Amyloses: The effect of ammonia pretreatment of the starch granules upon the molecular size of the amylose was investigated by measuring the molecular weights of amyloses from pretreated and non-pretreated samples. The two Zimm-plots (see figure 36 for Zimm-plot of NH_3-pretreated sample) were similar. The second virial coefficient is essentially zero for this solvent in the concentration range investigated ($5\text{-}20 \times 10^4$ g./ml.). Both Zimm-plots show distinct curvature. It is thought that this curvature is due to the amylose and is not an artefact introduced by either dust or faults in the alignment of the light-scattering photometer because of the following observations;

(i) The accuracy of the alignment of the light-scattering instrument is shown by the constancy of the scattered envelope from fluorescein solution (Section II).

(ii) Results were only accepted when the values of the scattering of the lowest concentration line were only altered by 10% or /

FIGURE 36



ZIMM–PLOT for TOTAL AMYLOSE in 0·33M KCl.

or less by the correction for solvent scatter.

(iii) The Zimm-plots for amylopectin were linear for the same relative degree of scattered light.

(iv) The curvature was unaffected by the use of various cells, including that of Banks (1960) in which back-scatter is considerably reduced.

(v) Exterpolation of the lower part of the Zimm-plot remained finite, and was constant in several solvents (0.33M KCl, 0.15M KOH, 1.0M KOH). Curvature due to dust is variable and tends to cause exterpolations to negative values.

(vi) The curvature was not eliminated by different clarification techniques.

The curvature can be accounted for by the theory of Benoit, Holtzer, and Doty (1954), who have shown that curved Zimm-plots can arise from solutions of stiff molecules when the distribution is sufficiently broad. Potato amylose from the sample of starch studied has a $\overline{M_w}$ -value of about 5×10^6, which confirms the high molecular weight indicated by the viscosity measurements (Dr. A.R. Proctor). The ratio of $\overline{M_w}$ to $\overline{M_n}$ shows the large width of the molecular weight distribution. The results, including the radii of gyration $(\rho_z^{-2})^{\frac{1}{2}}$ are shown in Table 5 .

TABLE 5 .

Results of Light-Scattering Measurements on Total Amylose Samples.

Pretreatment	$[\eta]$ *	$10^{-6}\overline{M_w}$	$10^{-6}\overline{Mn}$	$\overline{M_w}/\overline{M_N}$	$(\overline{\rho z}^2)^{\frac{1}{2}}$
NH_3	720	5.8	1.4	4.2	1990
None	610	4.2	0.8	5.2	1430

* in 0.15M KOH

It can be seen that ammonia pretreatment causes slightly more higher molecular weight material to be extracted.

The apparently extremely broad distribution shown by the light-scattering measurements could be due to the presence of a few percent of extremely high molecular weight particles, perhaps even stable aggregates (Compare Manley and Bengtsson (1958)). Indeed both Lewis and Smith (1957) and Procter (1965) have shown that amylose is heterogeneous. Procter (1965) has shown that the heterogeneity is due to a small, fast-moving peak, in the ultracentrifuge and that this high molecular-weight material is probably a branched amylose. The anomaly is not present in subfractions whose β-amylolysis limit is 100%.

Zimm-Plots of Amylose Subfractions. In an attempt to extend the work of Banks and Greenwood (1963), several linear amylose subfractions were investigated by light-scattering. In no case was a linear Zimm-plot of the type reported by Banks and Greenwood (1963) or Cowie (1961) (DMSO solution) found. The curvature was felt to be inherent in the samples for the reasons given above. On examination of the two above reports, it was noted that Banks and Greenwood used a limited angular range (35°-90°) and that Cowie applied a back-scattering correction and as a result only utilised angles $\gtrsim 45^{\circ}$. It was found with the present results, e.g. those in figure 36, essentially linear plots could be obtained at angles $\gtrsim 45^{\circ}$ by the application of the correction factor used by Cowie, but it is felt that this correction is not justifiable in the present work.

It /

It is suggested that the curvature is due to the molecular weight distribution of the samples, and arises through the inadequacies of the fractionation method. Until some method of proper fractionation is devised no precise hydrodynamic behaviour of amylose can be deduced; this may be the cause of some of the discrepancies in the literature.

The Conformation of Amylose in Aqueous Solution (See Section VII).

Procter (1965) has shown that there is a large increase in viscosity of amylose solutions in alkali especially at ca. 0.15M. This has been confirmed by measurements of the radii of gyration from curved Zimm-plots of the type shown in figure 36, for amylose in 0.33M KCl, 0.15M KOH, 1.0M KOH. In all three cases, the particle weight as determined from either exterpolation was constant within experimental error. The results are compared with Procter's viscosity results in Table .

Solvent	$[\eta]$	ρ_z
0.33M KCl	190	1010
1.00M KOH	450	1140
0.15M KOH	510	1300

These results confirm that the changes in viscosity arise from changes in molecular volume. The nature of this conformational change is discussed in Section VII.

SECTION VII.

NUCLEAR MAGNETIC RESONANCE STUDIES.

SECTION VII.

NUCLEAR MAGNETIC RESONANCE STUDIES.

INTRODUCTION.

Until relatively recently, direct studies on the conformations of carbohydrates and polysaccharides have been mainly confined to crystalline or amorphous states. However, with the advent of high-resolution nuclear magnetic spectroscopy, it has been possible to gain some insight into the conformation of the molecules in solution.

The first N.M.R. investigation of carbohydrates was by Lemieux *et al* (1958) on fully acetylated pentopyranosis and hexopyranoses. Later work on glucose and its derivatives in aqueous solution has been published by Lenz and Heeschen (1961) (several monosaccharides), Pasika and Cragg (1962) (highly branched dextrans), Rao and Foster (1963) (some small oligomers), van der Veen (1963) (glycosides of D-glucose and D-galactose) and Rao and Foster (1965a) (effect of alkali upon the conformation of small oligomers). More recent work has been concerned with the conformation of carbohydrates in dimethylsulphoxide solution. (Casu *et al* (1964), Rao and Foster (1965b), Casu *et al* (1965)).

All of the conformational studies by N.M.R. have been concerned with mono- or di- saccharides and the behaviour of the polymer in solution has been inferred from the behaviour of /

of its monomers. This arises because the peaks in the N.M.R. spectra of polymers are broad and diffuse, and fine details such as peak-splitting are lost because of (i) the spreading of the applied field by the small additional fields of the neighbouring nuclei and (ii) the lack of sufficient segmental motion within the polymer.

More resolved spectra could be obtained by the use of higher temperatures with consequently increased segmental motion but this facility is, at present, limited to the more powerful N.M.R. spectrometers.

It may be noted that the spectra mentioned above for the polymer, dextran, were poorly resolved from a configurational view-point but nevertheless were accurate enough to provide a measure of the ca. 40% branching present in the polymer.

It has been shown in the above-mentioned publications that in sugars

(i) the signal due to the anomeric proton appears at lower field than that of any of the other carbon-bonded hydrogen atoms

(ii) the signal for an equatorial anomeric proton occurs at a lower field than that for a corresponding axial anomeric proton and (iii) the dihedral angle between the anomeric proton and the proton on the adjacent carbon atom can be obtained from the magnitude of the splitting of the absorption peak due to the anomeric proton.

For (iii) calculations have been based on the Karplus (1959) equation /

equation as modified by Lenz and Heeschen (1961) i.e.

$$J = F\,[A\ \cos^2\phi - 0.28]$$

J = observed splitting

$F = 1.09 \pm 0.05$

$A = 8.5$ for $0^\circ \leqslant \phi \leqslant 90^\circ$ and $A = 9.5$ for $90 \leqslant \phi \leqslant 180^\circ$

Some criticism of the accuracy of this equation has been discussed by Hall (1964), but the equation still seems to be applicable to glucose-type spectra - at least on a first-order basis. (The accuracy of any angles quoted cannot, however, be expected to be greater than $\pm 5^\circ$.).

The Conformation of Amylose. The conformation of the amylose polymer in solution is very dependent upon the conformation of the monomers; e.g. if the glucose molecules adopt the C1 configuration (convention of Reeves (1949)) the amylose chain will assume a relatively rigid helix conformation (Hollo and Szejdli (1961)). Infra-red studies (Greenwood and Rossotti (1958), Casu and Reggiani (1964)) have indicated that glucopyranose rings in many starch derivatives assume a common shape, most probably C1. Of the many other chair and boat forms possible the more likely would be B1 or 3B, from stability considerations (Greenwood and Rossotti (1958)). Reeves has suggested (1954) (1958), that approximately half of the glucose residues in amylose exist in a boat conformation, and that the non-reducing unit of maltose possesses a boat conformation, a conclusion supported by Bentley (1959). All the N.M.R. data so far available /

available fail to support this conclusion.

EXPERIMENTAL.

All spectra were measured on the Perkin Elmer nuclear magnetic resonance spectrometer.

Samples for aqueous solutions were deuterated by freeze-drying three times from D_2O (99.9%) solution. In dimethyl-sulphoxide solution adequate deuteration was obtained by adding a few drops of D_2O to the N.M.R. sample (Casu et al (1965)).

τ-values are based on external (internal in case of dimethylsulphoxide solutions) tetramethyl silane standard or the internal water peak of neutral aqueous solutions (= 5.2τ, Lenz and Heeschen (1961)).

Most spectra were obtained from 15-20% solutions.

Glucose oligomers were obtained by chromatography of the products of the partial acid hydrolysis of amylose (Miss E.A. Milne and Dr. W. Banks).

RESULTS.

Neutral Aqueous Solution: Typical spectra are shown in figure 37. The splittings of the anomeric protons were measured from the average of several values at higher magnification (x6). The peak at 5.2 τ is assigned to residual water. The two peaks occurring at low field in the case of glucose are due to the anomeric proton in its two possible configurations (α - 4.74τ, β - 5.34τ). In the case of maltose, the third peak at low field (4.58τ) arises from the C_1 proton of the non-reducing glucose unit. Similar spectra, though less well-resolved due to /

to the scarcity of the samples, have been obtained for the glucose oligomers, G_3, G_5, G_6 and a mixture of G_7 and G_8 designated $G_{7/8}$ (see figure[37]). Samples of α-methyl glycoside and α-Schardinger dextrin were also measured in D_2O solution and the results are gathered in Table 6. It is interesting to note that in the case of the α-Schardinger dextrin only one peak appears at low field (4.84 τ) confirming the cyclic nature of the hexasaccharide.

TABLE 6.

N.M.R. Data (D_2O Solutions)

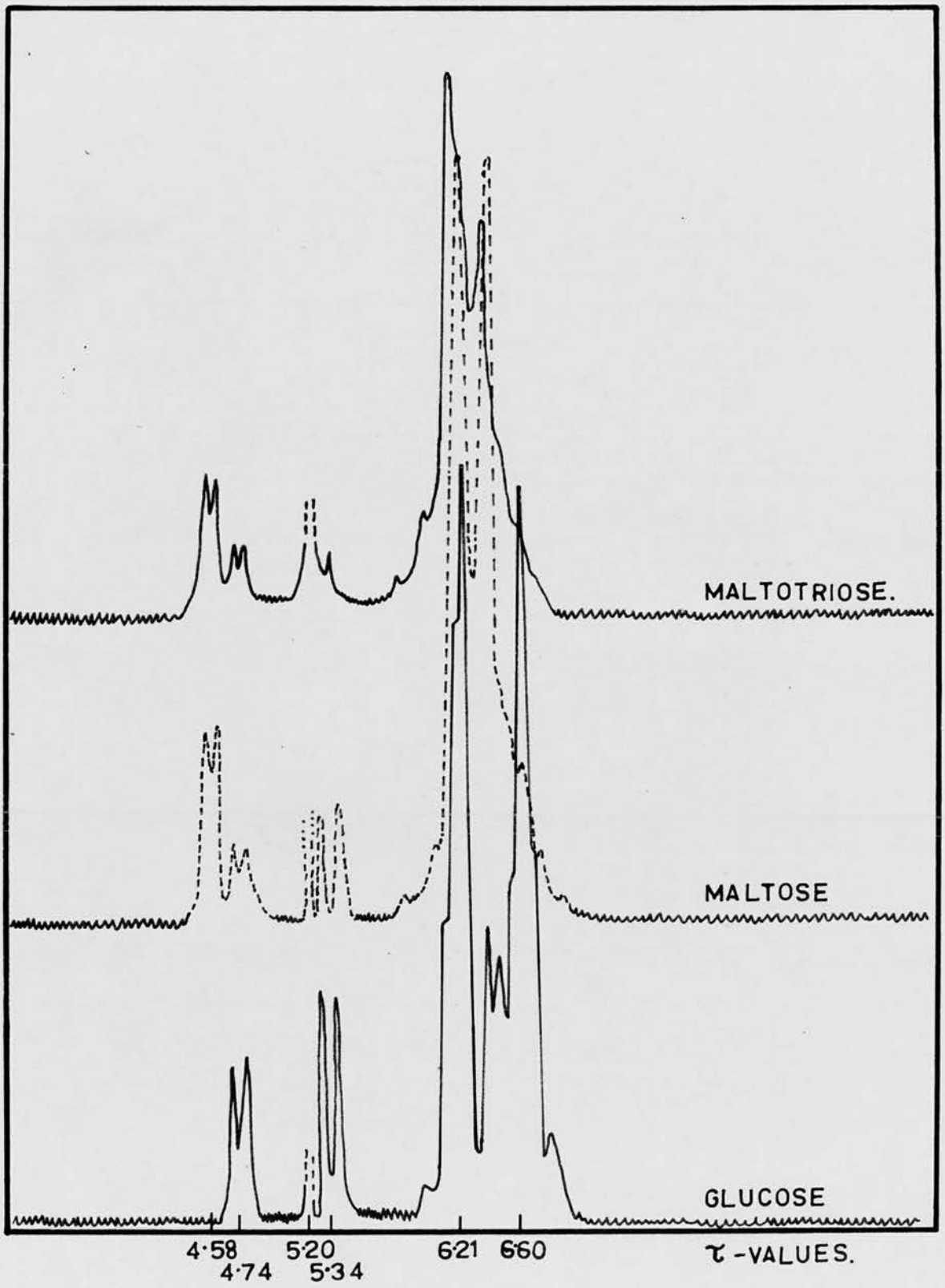
Sample	Chemical Shift (τ) of Anomeric Protons α	β	Coupling Constant J (c.p.s.)		Dihedral* Angle	
Glucose	4.74	5.34	3.1	7.3	52°	150°
Maltose	4.74	5.30	3.7	7.7	49°	152°
	4.58		3.0		53°	
α-Methyl Glycoside	5.11	-	3.1		52°	
α-Schardinger Dextrin	4.84	-	3.0		53°	
Glucose Oligomers G_3	4.65	N.R.	3.5		52°	
	4.51		3.1		50°	
G_5	N.R.	N.R.	-		-	
	4.50		2.9		54°	
G_6	N.R.	N.R.	-		-	
	4.48		3.1		52°	
$G_{7/8}$	N.R.	N.R.	-		-	
	4.50		3.0		53°	
Glycogen	N.R.	N.R.	-		-	
	4.50		N.R.		-	

N.R. - not resolved on spectra (low sample concentration)

* Calculated from Karplus equation.

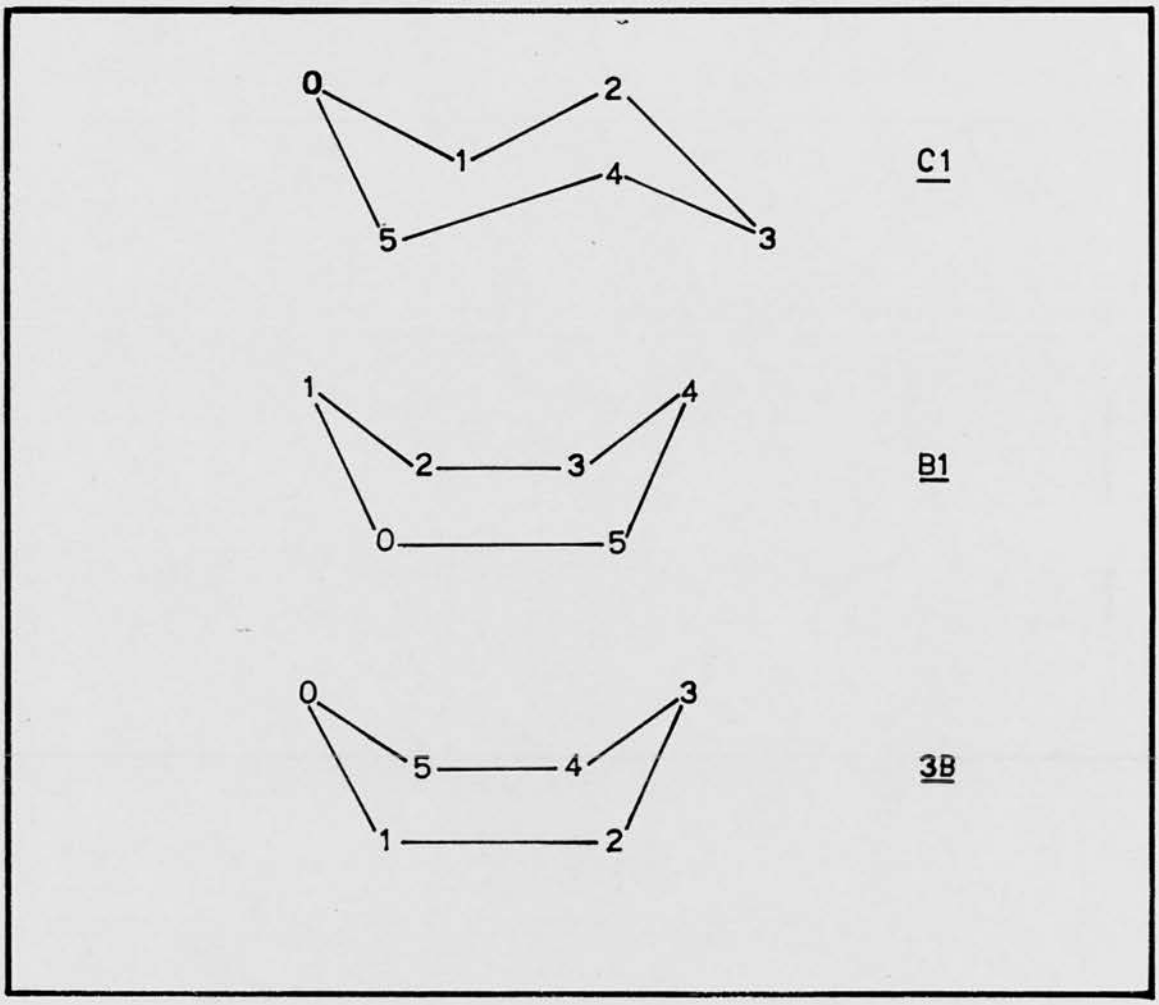
The similarity of the conformations in going from monosaccharide to polysaccharide is shown both by the small change in /

FIGURE 37



N.M.R. SPECTRA—D_2O SOLUTIONS.

FIGURE 38



REEVES' GLUCOSE CONFORMATIONS.

in τ-value (0.08 from maltose to glycogen) for C_1 protons attached to non-reducing glucose units and by the constancy of the coupling constant of this proton (3.0 $\pm$ 0.1 c./s. from maltose to $G_{7/8}$). This indicates that there is no abrupt conformational change in going from monosaccharide to polysaccharide and that consequently any conclusions drawn about the conformation of the monomer may be applied directly to the polymer.

From the calculated dihedral angles and the peak positions, it can be concluded that both glucose and the reducing unit in maltose is in the C1 configuration. The dihedral angles obtained for the non-reducing units of the oligomer up to $G_{7/8}$ suggest again that these units are in the C1 configuration. However this angle could also be in agreement with the B1 configuration but certainly not the 3B. It is, nevertheless, very unlikely that the glucose ring will occur in a perfect B1 form since the hydrogen atoms on C_1 and C_4 would be very close. Any twisting to relieve this repulsion would lead to a considerable change in dihedral angle. The B1 conformation is further disfavoured when the ready interconvertibility of B1 and 3B, as proposed by Freudenberg and Cramer (1950), is considered. Firstly, sharp dihedral splitting, as was observed, would not be expected if the dihedral angle was fluctuating and secondly the similarity of the peaks (between 6 and 7 τ) arising from the protons on carbon atoms other than C1, suggest no conformational change on going from glucose to glycogen.

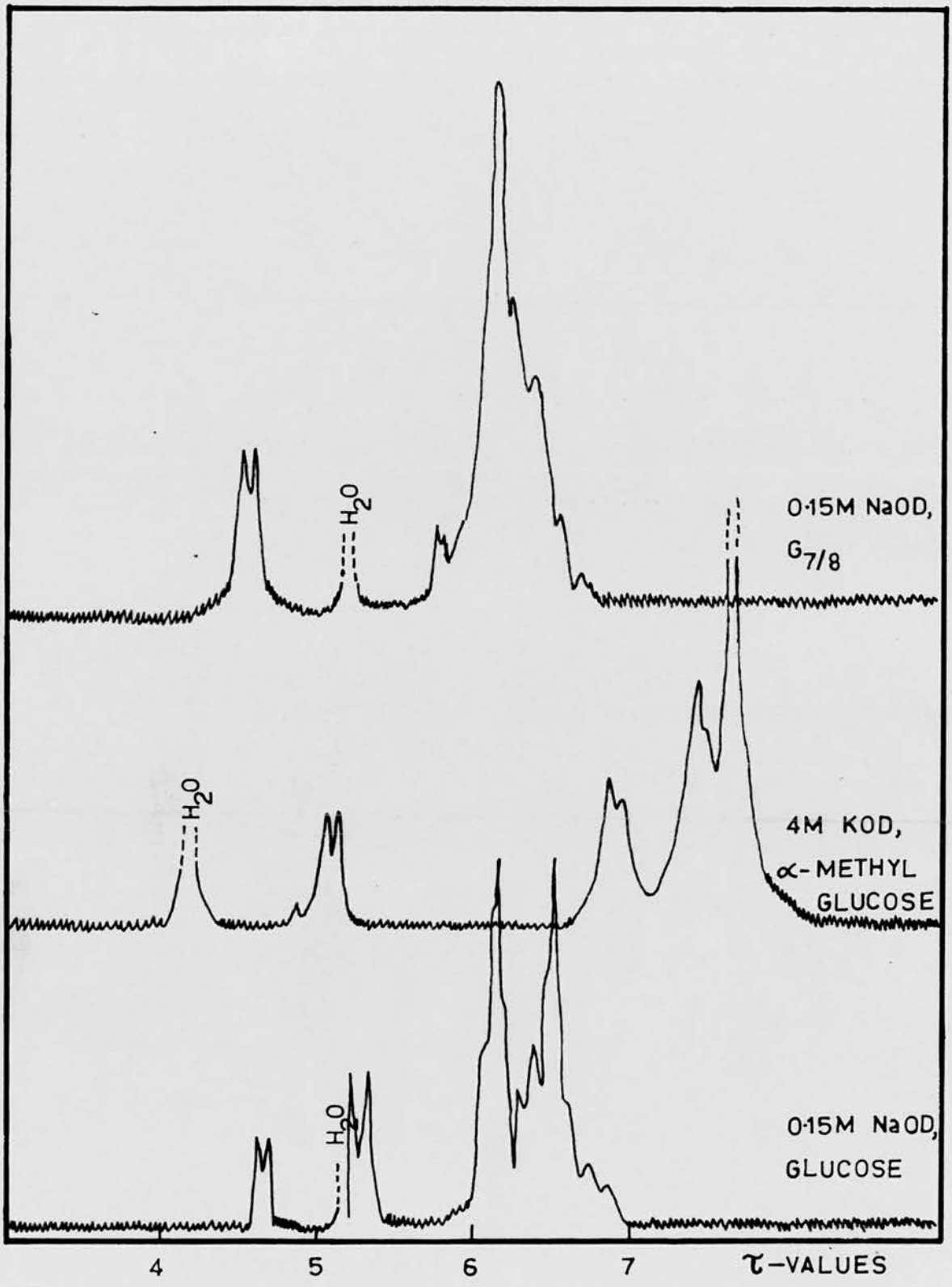
In /

In conclusion, it appears probable that all of the compounds mentioned in Table exist in the chair form, C1, in neutral aqueous solution. However other intermediate conformations, i.e. "half-chairs" and "half-boats", have been proposed (Bentley (1959)). Although it is not certain that these have a finite existence, it is possible that the sugars could exist in these intermediate forms. The bulk of these can be eliminated by the construction of models and only the remainder have suitable dihedral angles - HC3, HC4, HC5, 2B1, 1B2. (Bentley's convention). It is impossible to distinguish between these and the C1 form on the basis of N.M.R. until a full analysis of the spectra is possible.

Alkaline Solutions: Rao and Foster (1963) and Procter (1965) have noted that when amylose solutions are subjected to pH 10.5 there are gross alterations in hydrodynamic properties. To account for these, Rao and Foster suggested a helix-coil transition in amylose, while Procter agreed with the suggestion of Reeves (1954) that some of the rings in amylose exist in the B1 conformation in neutral solution and shift to the 3B conformation upon ionisation of the hydroxyls. A third possibility was proposed by Hollo et al (1961) to account for the marked decrease in optical rotation of alkaline solutions of glucose, maltose, and amylose. It was suggested that there was a shift from the C1 to the 3B conformation of the pyranose rings.

The suggestion of Reeves and Procter seems unlikely for the reasons given above. The conformational change proposed by Hollo /

FIGURE 39



N.M.R. SPECTRA-ALKALINE SOLUTIONS.

Hollo et al remains a possibility and could explain the helix-coil transition proposed by Rao and Foster.

Typical spectra are shown in figure 39, and full details are given in Table 8. It can be seen that the results are very comparable to those obtained in neutral aqueous solution.

The following conclusions may be drawn:-

(i) the conformation of carbohydrates is unaffected by alkali (0.15M to 4M) as compared with the conformation at neutral pH.

(ii) the conformation is unaltered by the addition of salt to dilute alkaline solutions.

(iii) in the case of glucose the anomeric equilibrium is displaced to favour the β-anomer practically exclusively in strong alkali. The ratio of α- to β- anomers in 0.15M alkali is substantially different from that in neutral solution.

(iv) the conformational change proposed by Hollo et al (1961) does not occur.

(i) and (iv) agrees with the recently published work, in 2M alkali, of Rao and Foster (1965a).

(ii) indicates that the abrupt hydrodynamic changes noted in amylose for solvents of this type (Procter (1965)) are not due to monomeric conformational changes.

Rao and Foster (1965a) have proposed that the differences in specific rotation, $[\alpha]_D$, observed for amylose in neutral solution and in 1M alkali (+40.0) can be accounted for by the increase in rotational freedom about the α-1→4 glycosidic bonds /

bonds in alkaline solution. They also suggest that changes in the optical rotation of the free sugars D-glucose and D-maltose are due to a shift of the anomeric equilibrium towards the β-form in alkali.

This latter suggestion has been confirmed both by the measurements in 4M alkali (practically total loss of α-anomer, as Rao and Foster found for 2M alkali) and in 0.15M alkali. The pH of the dilute alkali (12.5) is such that the changes in optical rotation have only partially occurred. It can be seen from Table that the change in optical rotation is closely connected to the percentage of the α-anomer.

TABLE 7.

Correlation of Optical Rotation Data (Rao and Foster (1965a)) and % of Anomers.

	H_2O		0.15M alkali		4M alkali	
	O.R.*	%α≠	O.R.*	%α ≠	O.R.*	%α ≠
Glucose	0	42%	+19	32%	+27	5%
Maltose	0	39%	+8	36%	+43	5%

* Change in optical rotation, $[\alpha]_{436}$, as compared to neutral solution.

≠ % content of α-anomer estimated from peak areas.

Both this data and the apparent consistency of the monomer configuration in a variety of sizes and degrees of polymerisations, indicates, in agreement with Rao and Foster, that the large hydrodynamic changes observed in alkaline solutions of amylose are due to increase in the rotational freedom of α-1→4 glycosidic /

TABLE 8 .

N.M.R. Data: (Alkaline Solutions).

Sample	Solvent	Chemical Shift (τ) of Anomeric Protons	Coupling Constant J (c.p.s.)
Glucose	0.15M NaOD	4.76, 5.34(β)	2.8, 7.2
	4M KOD	- 5.30(β)	6.9
Maltose	0.15M NaOD	4.59, 4.78	3.0, 3.3
	0.15M NaOD +2.5M NaCl	4.60 N.R.	3.0
α-methyl-glycoside	4M KOD	5.16	2.8
α-Schardinger dextrin	4M KOD	4.80	3.0
Glucose Oligomers G_5	0.15M NaOD	4.59	3.0
	0.15M NaOD +2.5M NaCl	4.59	3.0
	0.15M NaOD + 4M NaCl	4.59	3.0
G_6	0.15M NaOD	4.59	2.8
$G_{7/8}$	0.15M MaOD	4.59	3.0

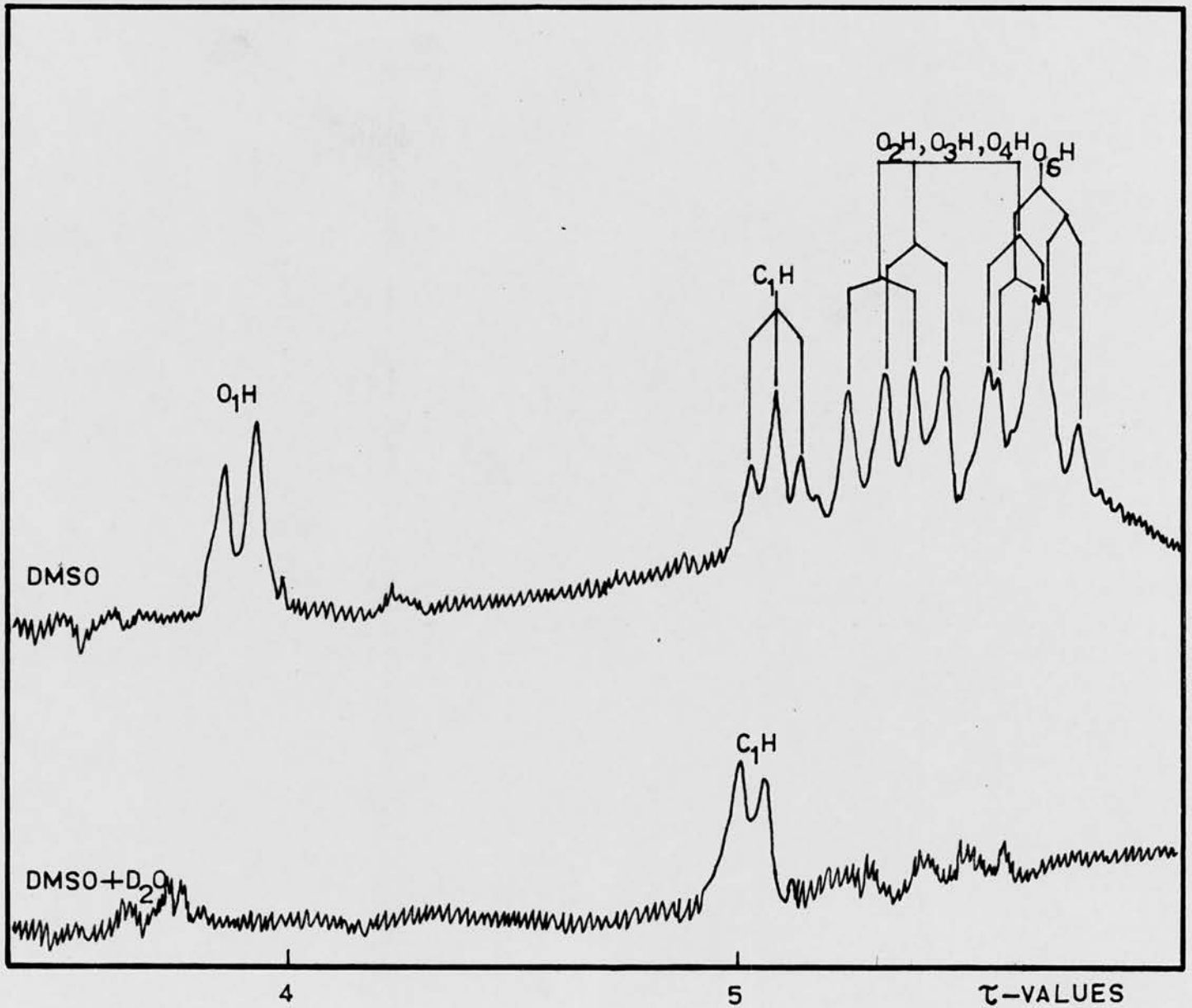
TABLE 9 .

N.M.R. Data: (DMSO Solutions).

Sample	Chemical Shift (τ) of α-Anomeric Protons	Coupling Constant J (c.p.s.)
Glucose	5.07	3.0
Maltose	4.96, 5.60	2.8
Glucose Oligomers		
G_3	4.96	N.R.
G_5	4.95	N.R.
G_6	4.95	2.5
$G_{7/8}$	4.96	2.6

N.R. - not resolved on spectra.

FIGURE 40



N.M.R. SPECTRA of GLUCOSE in DMSO — ASSIGNMENTS after CASU et al. (1965).

glycosidic bonds with the amylose molecule.

Dimethylsulphoxide (DMSO) Solutions: DMSO is a good sugar solvent and has been used for physicochemical studies on amylose (Everett & Foster (1959)). The spectra of glucose and other simple sugars has been reported by Casu et al (1964). These authors assigned the hydroxyl and anomeric proton resonances ascribing the peak at 5.07τ to the anomeric proton (Casu et al (1965)).

In the present work, the peak due to the anomeric proton was identified by (i) its appearance at low field; (ii) the disappearance of all other low-field peaks due to hydroxyl proton resonances when the sample is deuterated (see figure 40).

By this technique it was shown that the peak at 3.82τ was wrongly assigned by Rao and Foster (1965b) to the anomeric proton and that consequently the results show no evidence of complex formation as they had suggested.

Table 9 gives details of the results. It can be seen that, although the chemical shifts are slightly greater than those in D_2O, the results for the splitting of the anomeric peak are substantially the same. Therefore in this solvent also, the glucopyranose rings are most probably in the C1 conformation.

CONCLUSION.

Glucose monomers in the free state or in polymer molecules appear to assume the same conformation. This conformation is apparently independent of solvent. It is probable that the conformation is C1.

TABLE 10 .

List of Aqueous Solvents over which the Splitting of the α-anomeric Proton remained effectively constant.

($J \doteqdot 3.0$ c.p.s.)

Substance		Solvent
Glucose		D_2O
Maltose		D_2O
α-methyl-glycoside		D_2O
α-Schardinger Dextrin		D_2O
Glucose Oligomers:	G_3	D_2O
	G_5	D_2O
	G_6	D_2O
	$G_{7/8}$	D_2O
Glycogen		D_2O
Glucose		0.15M NaOD
Maltose		0.15M NaOD
Glucose Oligomers	G_5	0.15M NaOD
	G_6	0.15M NaOD
	$G_{7/8}$	0.15M NaOD
Maltose		0.15M NaOD+2.5M NaCl
Glucose Oligomers	G_5	0.15M NaOD+2.5M NaCl
	G_5	0.15M NaOD+4M NaCl
Glucose		4M KOD
α-methyl Glucose		4M KOD
α-Schardinger Dextrin		4M KOD

SECTION VIII.

THE KINETICS OF α-AMYLOLYSIS.

SECTION VIII.

THE KINETICS OF α-AMYLOLYSIS.

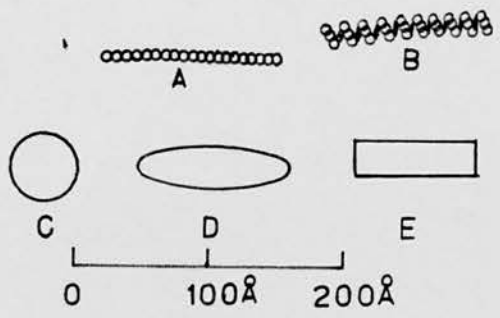
In conjunction with studies on the extraction of α-amylases by MacGregor (1964), an attempt was made to measure the relative rates of attack of these highly purified enzymes on different substrates by the light-scattering technique.

α-amylases are obtained from a wide variety of sources (animal, plant, and microorganisms) and may be expected to differ more from each other than do the more specific β-amylases which are only obtained from higher plants. The action of α-amylase on amylose has generally been considered to be random such that the long chain is attacked indiscriminately throughout its length with the production of shorter chains (Myrbäck, (1948)). These in turn are broken down until nothing remains but small oligosaccharides. In contrast to the β-amylases, the α-amylases are able to cleave branched substrate molecules between the branches.

The relative size of amylose molecules and starch chains is compared in Figure 41a. (after French, 1957). The dimensions are based upon the ultracentrifugal and diffusion measurements of amylases and other low molecular weight proteins by Cohn and Edsall (1943), together with X-ray data relating to the size of glucose units in starch chains. No matter which of the likely shapes the protein molecule assumes, it will be highly compact in comparison with chain polymers of comparable molecular weight. On the other hand amylose chains are probably coiled in an irregular manner.

Amylopectins /

FIGURE 41



A 20-unit amylose chain

B 60-unit amylose helix

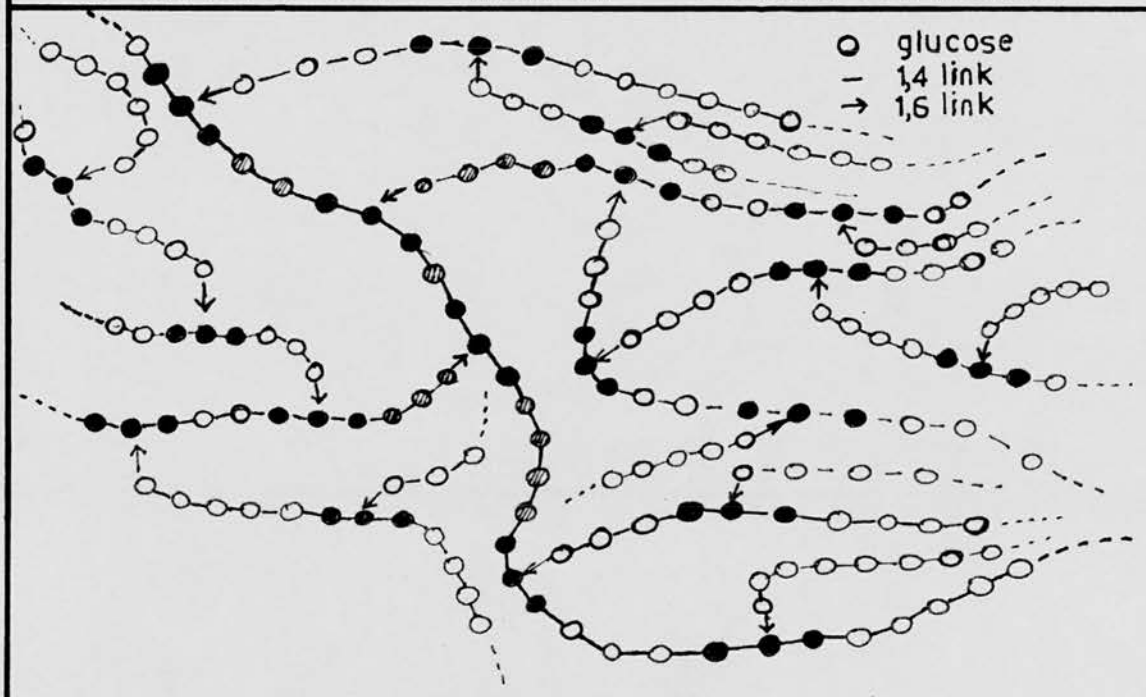
Size of amylase (M.Wt. 45,000) if

C spherical

D an elongated ellipse

E a bundle of 7 helices

(a) RELATIVE SIZES of AMYLOSE and AMYLASE MOLECULES.



ATTACK
- IMPOSSIBLE STERICALLY.
- UNLIKELY because of density of surrounding chains.
- FREE, RANDOM.

(b) RELATIVE PROBABILITIES of ATTACK by AMYLASE on a centre portion of an idealised BRANCHED GLUCAN. (SEE TEXT).

Amylopectins and glycogens, having molecular weights in the millions, would be far larger than the chains shown and would extend in bush-like fashion over a space of hundreds of Angstrom units (see, for example, the root-mean-square end-to-end distances quoted in Section III for amylopectins).

EXPERIMENTAL.

α-amylases.

α-amylases isolated from soya bean, barley, and malted-barley were provided by Dr. A.W. MacGregor.

Substrates.

Amylose and amylopectin were prepared by the standard techniques. Glycogen was isolated from rabbit liver by extraction with TCA (see Section V). The β-limit dextrins were prepared by digestion of the amylopectin or glycogen with β-amylase. All substrates were dissolved in distilled water and their concentrations adjusted to the same value to keep the enzyme-substrate ratio constant.

Light-scattering.

The substrates were filtered through Millipore filters (pore diameter 1.2μ for amylopectin, 50mμ for glycogen and amylose) until dustfree. Measurements were made at constant temperature ($20^\circ \pm 1^\circ C$) using the mercury green line (546mμ) and angles 0°, 45°, 90°, and 135°.

Samples of the enzyme were added directly to the light-scattering cell from an Agla syringe. The enzyme preparations were so active that only very small volumes were required (of the order of 0.01 ml.). The decrease in scattered light was followed, over a period of hours, from the addition of the enzyme.

RESULTS./

RESULTS.

Initially results were treated simply as a measure of the relative decrease of 90°-scatter at any one time compared to the 90°-scatter of the original substrate. This resulted in plots like those of Banks et al (1960) (See Figure 42). However the curvature of this type of plot made any estimate of the relative ratio extremely hazardous.

A more satisfactory method of evaluating the data was to plot log $(R_{90})o/(R_{90})t$ versus t, where $(R_{90})o$ is the 90° scattering (corrected for dissymetry) at time = 0 and $(R_{90})t$ is the corresponding scatter at time = t. These graphs have an initial linear portion before the onset of curvature. It was therefore easier to get some idea of the relative rates of enzyme attack on the different substrates by these plots. Now if the original concentration of substrate is 'a' and an amount 'x' is degraded at time 't' i.e. the amount left is (a - x), then the original scattering at 90°, corrected for dissymmetry of scatter, is

$$R_{90} \propto \overline{Mw} \times a,$$

and since only small degrees of conversion are being considered (ca. 1 link in 6000 links), it is likely that the weight-average molecular weight will remain effectively constant over the time-range. Hence,

$$\frac{a}{a-x} = \frac{(R_{90})o}{(R_{90})t}$$

Thus,

$$\log \frac{(R_{90})o}{(R_{90})t} = \log \frac{a}{a-x},$$

and /

and for simple first-order reactions this is linear with respect to time. A typical graph is shown in Figure 43. The linearity of these plots does not, however, prove that the enzyme-substrate reaction is first-order (I am indebted to Dr. W. Banks for bringing this to my attention).

First order kinetics for random hydrolysis are such that

$$\log \frac{a}{a-x} \quad \text{versus t is linear}$$

where a = number of bonds available to hydrolysis at t = 0

x = number of bonds lost by time t.

For low conversions, x is small, hence

$$\log \frac{a}{a-x} = \frac{x}{a}$$

Now x = number of bonds broken

$$= no \left(\frac{Po}{Pt} - 1\right)$$

where no = number of molecules initially

Po = initial degree of polymerisation

Pt = degree of polymerisation at time t.

Obviously a = noPo

$$\therefore \frac{x}{a} = \left({}^{Po}/_{Pt} - 1\right)/Po$$

$$= \frac{1}{Pt} - \frac{1}{Po}$$

$\therefore \frac{1}{Pt} - \frac{1}{Po}$ versus t is linear for first-order reactions.

But zero-order kinetics for random degradation require that

$\frac{1}{Pt}$ versus t is linear (Vink, 1963).

Thus for low conversions it is impossible to decide between the /

the two mechanisms.

The results have been evaluated on the first-order basis and are detailed in Table II .

TABLE II .

Rates of Enzyme Attack on Various Substrates.
(Relative to Amylose)

Substrate	α-Amylase		
	Soya Bean	Malted Barley	Barley
Amylose	1.00	1.00	1.00
Amylopectin	0.58	0.68	0.67
Amylopectin β-Limit Dextrin	0.46	0.41	0.40
Glycogen	0.32	0.23*	0.04
Glycogen β-Limit Dextrin	0.10	0.09*	0.02

TABLE 12.

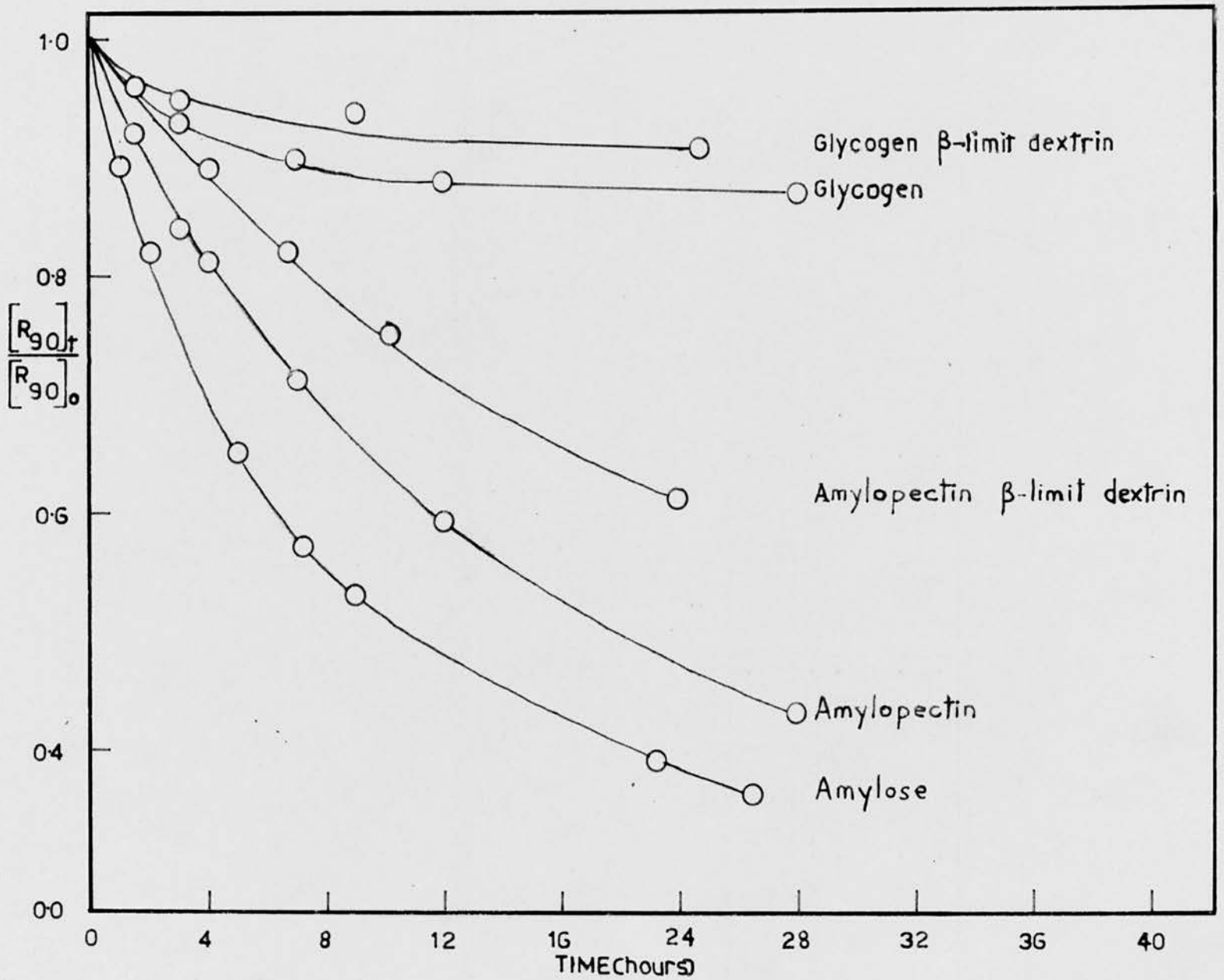
Theoretical Rates of enzyme Attack on Various Substrates.
(Relative to Amylose)

Substrate	% branching	No. of Groups unavailable (3 per branch point) Relative to Amylose	Material in External Chains /	Relative Rates
Amylose	0	1.00	100%	1.00
Amylopectin	4	0.88	60%	0.53
Amylopectin β-Limit Dextrin	9	0.73	48%	0.35
Glycogen	10	0.70	40%	0.28
Glycogen β-Limit Dextrin	20	0.40	25%	0.10

* Activity of the enzyme X 7

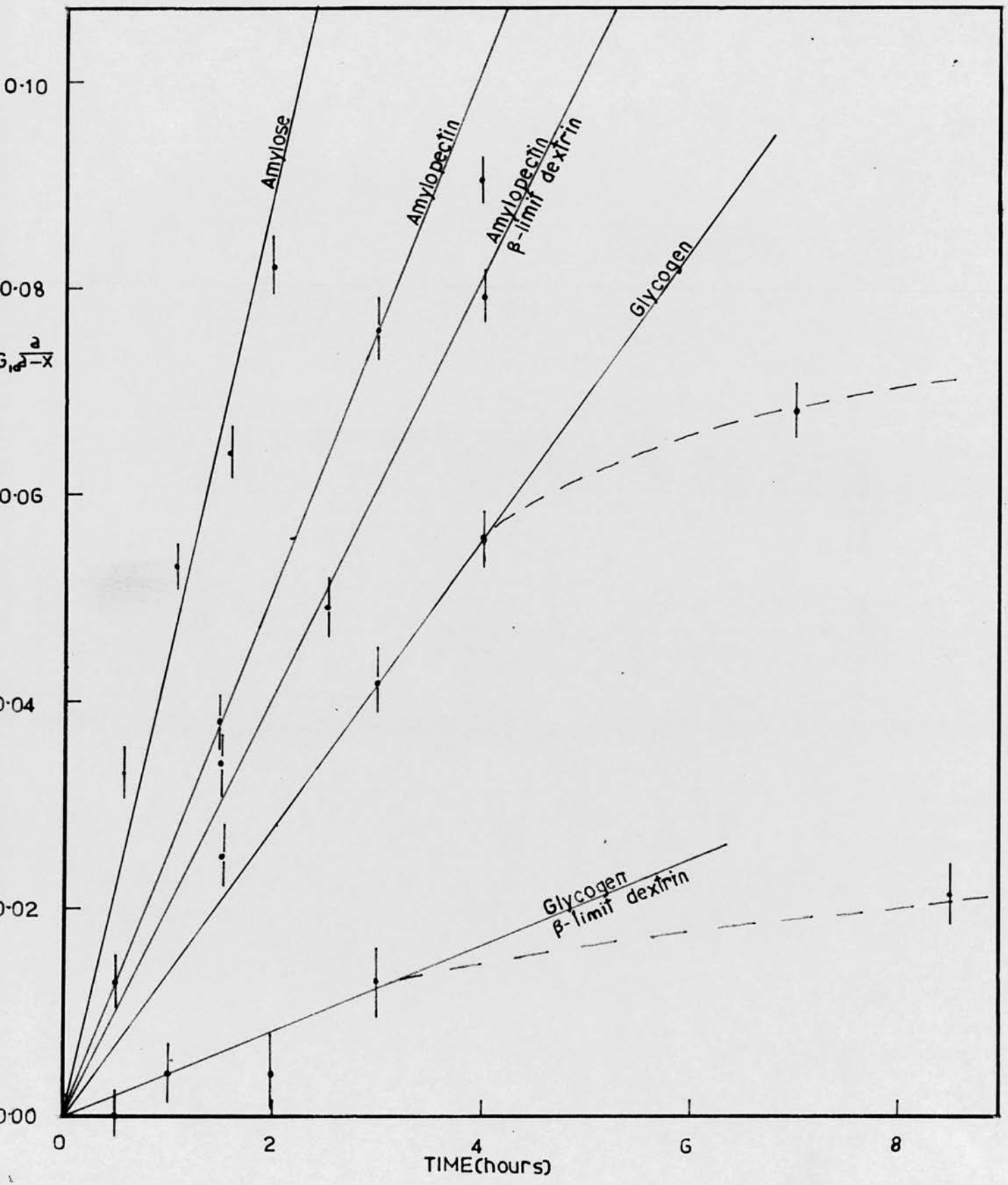
/ From the statistical calculations of Erlander (1959)

FIGURE 42



Action of SOYA BEAN ∝-AMYLASE on various substrates as shown bylight-scattering.

FIGURE 43



First-order reaction rate plot for SOYA BEAN ∝-AMYLASE.

Discussion.

From the results, it seems clear that the mode of action of the three enzymes are essentially similar. Soya bean α-amylase appears to be able to penetrate the highly-branched glycogen and glycogen β-limit dextrin molecules with greater ease than the other two enzymes. This would suggest that it was being less sterically hindered probably because of a smaller molecular size.

The relative rates of attack of the enzyme can be predicted from the number of sites on the molecule available for enzymic attack. Firstly sites at, and immediately adjacent to, 1→6-linked groups will be stereochemically unlikely to be attacked i.e. 3 groups per branch point. It is also likely that in highly branched structures exterior chains will be preferentially attacked and hence the relative rate will be dependent on the amount of material in the external chains. The theoretical rates and their derivation are shown in Table 12.

The theoretical relative rates agree reasonably well with the measured experimental values. Again, it appears that the barley and malted barley α-amylase are unable to penetrate the denser structures as well as the soya bean α-amylase, suggesting a difference in molecular size.

CONCLUSION.

The action pattern of the three α-amylases on various substrates has been compared and shown to be essentially similar. The rates of enzyme attack on branched substrates relative to amylose have been predicted from a consideration of the number of sites available for random attack (See Figure 41b). It appears that soya bean α-amylase is of smaller molecular size than barley - or malted barley α-amylases.

SUMMARY

SUMMARY.

Physical techniques have been applied to the study of the starch polysaccharides and glycogen; these include ultracentrifugation, viscosity, light-scattering and nuclear magnetic resonance.

The behaviour of amylopectin solutions has been shown to be determined by two effects. The first, at low concentrations ($< 2 \times 10^{-3}$ g/ml), is due to the phosphate groups present in amylopectin and confers typical polyelectrolyte behaviour on the amylopectin molecule. The second effect, which occurs at higher concentrations, is apparently the formation, by some of the amylopectin molecules, of colloidal aggregates. Amylopectin has been converted to its "free acid" form by ion-exchange resins.

The results of present studies on starch biosynthesis are inconsistent with the current biosynthetic theories. From a consideration of the literature and the present work a new theory has been proposed.

Studies on the extraction of glycogen from liver, by various methods, have shown that the broadest distribution of molecular sizes are extracted by a technique which uses a phenol/water system rather than the classical 30% sodium hydroxide solution, or some more modern methods. The effect of degrading agents upon glycogens of various sizes has been studied by the effect of /

of these agents upon the sedimentation-coefficient distributions of differing glycogens. Molecular-weight distributions have been derived for three broad distribution glycogens and their β-amylolysis dextrins, and these indicate that all the molecular weight species of the glycogens are attacked to the same extent by the enzyme. A "sub-unit" hypothesis has been proposed for high molecular weight glycogen.

Studies on the N.M.R. spectroscopy of glucose oligomers have shown that the conformation of the glucose monomer in a long chain polymer such as amylose is unaffected by alkaline conditions (0.15M - 4M) or by the addition of salt of the dilute alkali. The gross hydrodynamic changes which occur under these conditions are ascribed to greater freedom of rotation about the glycosidic α - 1 $\rightarrow$ 4 bond. It is considered likely that the conformation adopted is C1. The same conclusions have been shown to apply for dimethylsulphoxide solutions of the oligomers.

Number-average molecular weights have been derived from light-scattering measurements on unfractionated amylose solutions.

The action pattern of three α-amylases has been compared by light-scattering measurements of their action on various substrates. The rates of enzyme attack on branched substrates relative to amylose have been predicted from a consideration of the number of sites on the polysaccharide molecules available for attack by the enzyme.

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